

**A metabolic approach towards optimising equine
in vitro maturation**

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of Liverpool for the degree of
Doctor of Philosophy
by

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Abstract

In the horse, there is a paucity of species-specific IVM optimisation and energy metabolism pathways in COCs during IVM are undefined. Studies were undertaken to characterise glucose, pyruvate and lactate (GPL) and oxidative metabolism (OXPHOS) in equine COCs during IVM and further investigate these parameters under varying clinical conditions. Gene expression in single oocytes, cumulus cells and blastocysts was also explored. Novel technology for the horse; enzyme-linked metabolic assays, the Seahorse XFp Analyser, PolyA PCR and Primovision™ were employed to generate data. Data demonstrated that COCs had high glucose consumption, accounted for by lactate production (glycolytic index ~100%). Up to 24% COCs depleted all available glucose during IVM, which was not associated with a change in maturation or cleavage rate. COCs produced and secreted pyruvate into the medium. Glucose/lactate (GL) metabolism was independent of cumulus/meiotic status but correlated to COC DNA content. Pyruvate production was independent of DNA content. GPL metabolism displayed a temporal pattern; GL increased during 10-20 h and GL decreased and pyruvate production increased during 20-30 h. Oxygen consumption rate (OCR) was high, demonstrating OXPHOS plays a larger role in equine COC energy metabolism than in other species. Examination of mitochondrial function showed that ~50% of OCR was coupled to ATP production, ~35% was non-mitochondrial and 17% due to proton leak and did not vary over time. In contrast, spare capacity fell from 85% to 5% over the course of IVM. Addition of 0.15 mM pyruvate increased glucose consumption and at 5% oxygen, 0.15 mM pyruvate decreased pyruvate production. Pyruvate concentration (0-0.5 mM) had no effect on OCR or mitochondrial function. Addition of 0.5 mM pyruvate to 17 mM glucose medium increased expression of G6PD in cumulus cells and resulted in a switch to pyruvate consumption. Glucose concentration (5.6/17 mM) had no impact on developmental, morphokinetic, GPL or OCR measurements but the proportion of OCR coupled to ATP and non- mitochondrial processes were decreased and increased respectively. Taken together, these results demonstrate the plasticity of the equine COC in terms of substrate utilisation but that this plasticity incurs a change at the metabolic level that warrants further investigation.

List of Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AR	Antimycin/Rotenone
ART	Assisted reproductive technologies
BMP15	Bone morphogenetic protein 15
EGF	Epidermal growth factor
BSA	Bovine serum albumin
CNP	C- type natriuretic peptide
CPT1	Carnitine paltoyltransferase-1
Cp	Oocyte with compact cumulus
COR	COC with corona only
CORE	Consumption and Release assays
CL	Corpus luteum
COC	Cumulus oocyte complexes
CV	Coefficient of variation
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CZB	Chatot Ziomek Bavister medium
DCA	Dichloroacetic acid
DEG	Degenerating oocyte
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
DOHAD	Developmental origins of health and disease
EDTA	Ethylene diamenetetraacetic acid
EGA	Embryonic genome activation
EH	Earle's hank's medium
EGF	Epidermal growth factor
EPSP	4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid
Ex	Oocyte with expanded cumulus
FAO	Fatty acid oxidation

FCCP	Carbonyl cyanide4-(trifluoromethoxy)phenylhydrazone
FBS	Fetal bovine serum
FADH ₂	Flavin adenine dinucleotide
FSH	Follicle stimulating hormone
GVBD	Germinal vesicle breakdown
GLUT	Glucose transport proteins
GL	Glucose, lactate metabolism
GSH	Glutathione
GnRH	Gonadotropin releasing hormone
GPL	Glucose, pyruvate, lactate metabolism
GPX	Glutathione peroxidase
GDF9	Growth differentiation factor 9
GV	Germinal vesicle
HBP	Hexosamine biosynthesis pathway
HK	Hexokinase
HG	High glucose
HG-HP	High glucose- high pyruvate
HIF	Hypoxia inducible factor
ICM	Inner cell mass
IGF	Insulin like growth factor
IVP	<i>In vitro</i> embryo production
IVF	<i>In vitro</i> fertilization
IVM	<i>In vitro</i> maturation
ITS	Insulin transferring selenium
IL1 β	Interleukin 1 β
ICSI	Intracytoplasmic sperm injection
LH	Luteinising hormone
MPF	Maturation promoting factor
MII	Metaphase II
M199	Medium 199
MAPK	Mitogen activated protein kinase
MNSOD	Manganese superoxide dismutase

mtDNA	Mitochondrial DNA
NADH	Nicotinamide adenine dinucleotide
NOD	Non-obese diabetic
NMR	Nuclear magnetic resonance
OCR	Oxygen consumption rate
OXPPOS	oxidative phosphorylation
PABP	Poly A binding protein
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PPP	Pentose phosphate pathway
PD3	Phosphodiesterase 3
PFK	Phosphofructokinase
PRPP	Phosphoribosyl pyrophosphate
PKA	Protein kinase A
PGE ₂	Prostaglandin E ₂
PT	Pipette trimmed
PVP	Polyvinylpyrrolidone
QC	Quality control
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RT	Reverse transcriptase
SO	Super oxide
SOF	Synthetic oviductal fluid
SRY	Sex determining region Y
STZ	Streptozotocin
TCM 199	Tissue culture medium 199
TE	Trophectoderm
TVA	Transvaginal aspiration
TCA	Tricarboxylic acid cycle
UPC	Uncoupling proteins
UTJ	Uterotubular junction
VT	Visually trimmed

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Chapter 1: General Introduction

1.1 Overview

Horses have been bred for pleasure and competition purposes for thousands of years. In the last century, breeding by artificial insemination has become commonplace for many breeds with the notable exception of racing thoroughbreds, for which the use of assisted conception is specifically excluded by the regulatory authorities. While artificial insemination allows the wider dissemination of valuable genetics traits, in addition to many management benefits, it relies on a functional tubular female reproductive tract and adequate stallion fertility in order for an offspring to be produced. Conversely, *in vitro* systems bypass the reproductive tract and can utilise only a single spermatozoon, thereby allowing offspring to be produced from genetic combinations that would otherwise be lost.

Regardless of whether pregnancies are derived from natural or *in vitro* embryo production (IVP) systems, a good quality oocyte is a prerequisite for embryo development and production of a healthy offspring. While intrinsic factors related to the donor mare and donor follicle can directly influence oocyte quality before collection, *in vitro* procedures leading up to the fertilisation process, namely *in vitro* maturation (IVM) as performed in equine IVP, also have the capacity to affect oocyte developmental potential, and thus require optimisation. In the horse, IVP is relatively novel. While *in vitro* culture systems are capable of producing viable offspring in the hands of a few specialist centres, there has been a notable paucity of species-specific optimisation reported. This lack of detailed study not only leads to a loss of efficiency in the IVP process but also creates the potential for undesired downstream epigenetic effects on the resulting offspring.

Across the domestic species and man, a 'metabolic approach' has proved useful in directing the optimisation of *IVP* systems. Studying metabolism allows species-specific requirements to first be identified and then the culture system to be subsequently tailored to meet those needs. In the horse, the energy metabolism pathways in use by cumulus oocyte complexes (COCs) during IVM, and the preferred substrates for optimal and efficient ATP production and mitochondrial function, are unknown.

1.2 The reproductive biology of the mare

Several aspects of the reproductive biology of the mare are unique to the species and highly relevant to the clinical application of assisted reproductive technologies (ARTs). The summary provided below highlights these elements in context of the general reproductive biology of the mare.

1.2.1 Follicular development and cyclicity in the horse

The mare is a long day, seasonally polyoestrus (~April-October) breeder with an oestrous cycle length of approximately 21 days (Ginther, 1992). Ovarian cyclicity in the mare is entrained to photoperiod and the onset of the breeding season is mediated by decreased durations of nocturnal melatonin secretion during the longer days of spring. Spring changes in circadian melatonin profiles promote increased frequency of pulsatile gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus. These pulsatile GnRH secretions directly stimulate the release of follicle stimulating hormone (FSH) from the anterior pituitary. Ovarian follicles recruited under the influence of this FSH grow as a single cohort (known as 'new wave' follicles) until the point of selection for dominance and deviation, which occurs when the largest ovarian follicle attains a diameter of 21-23mm (Ginther et al., 2004). As mares are generally monovulatory, this is the point at which a single follicle will usually assert its dominance (Ginther et al., 2004). The dominant follicle(s) secretes oestrogen and inhibin, which feedback negatively to decrease FSH release from the pituitary. Related to this, the remainder of the follicle cohort undergoes atresia. The dominant follicle grows to upwards of 40 mm in diameter before ovulation, one of the largest periovulatory follicles in the animal kingdom. Oestrus is long and variable (most commonly between 5-7 days) and is characterised by a prolonged and gradual increase in peripheral luteinising hormone (LH) concentrations, which peak 1-2 days after ovulation (Ginther, 1992; Figure 1.1). This prolonged and gradual LH rise differs from the sharp LH peak found in man and most domestic species, but as for other species, it is recognized as the trigger event which brings about changes within the oocyte and follicle to culminate

in ovulation and luteinisation of follicular cells essential for the establishment of the corpus luteum (CL).

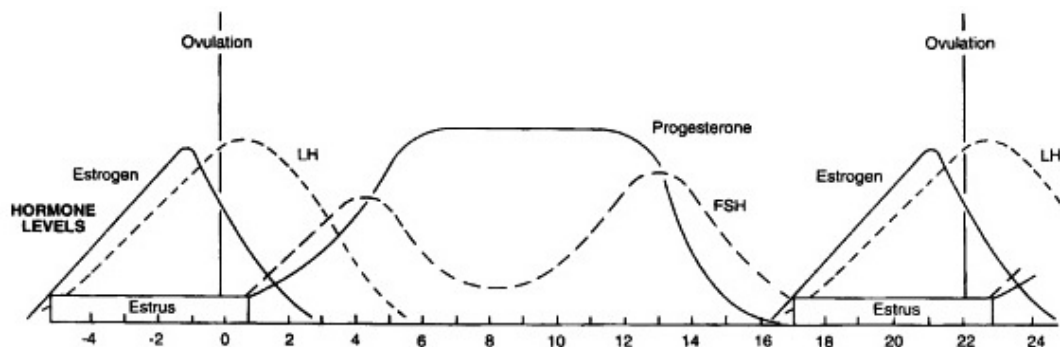


Figure 1.1: *Hormonal profile of the reproductive cycle of the mare (Adaptation of diagram by Daels and Hughes, 1993)*

The CL synthesises and secretes progesterone, which can be detected in the peripheral circulation 0-2 days post-ovulation (Hinrichs et al., 1988). Luteolysis occurs in the absence of pregnancy on days 14-16 of the cycle, due to PGF2 α release from the endometrium (Douglas and Ginther, 1976)

1.2.2 Reproductive anatomy of the horse

The gross anatomy of the mare's ovaries is essentially "inverted" in comparison to those of humans and other domestic species. The ovary is surrounded by a thick layer of connective tissue over all but one section, such that ovulation can only occur at a single point, named the ovulation fossa (reviewed by Gastal, 2011). The oviduct of the mare is also unique in that the uterotubular junction (UTJ) allows only selective transport of viable embryos. This demarcation creates two distinct environments (oviduct and uterus) to which the early embryo is exposed.

1.2.3 *In vivo* fertilisation and early embryo development in the horse

Ovulated oocytes enter the oviduct surrounded by follicular cumulus cells and remain viable for a period of at least 6-12 hours before they begin to degenerate (Ginther, 1992). Fertilisation takes place in the ampullary-isthmus junction of the oviduct. Fertilisation rates *in vivo* are upwards of 90% (Bezard et al., 1989) with the first cleavage occurring at approximately 24 hours post-ovulation. Embryonic development to the morula stage occurs within the oviductal environment and viable embryos are transported through the UTJ between Day 5.5-6 post-ovulation (Freeman et al., 1991). This selective transport is attributed to the secretion of Prostaglandin E₂ (PGE₂) by the developing embryo (Weber et al., 1991). In practical terms for research purposes, this prevents access to the early embryo until about Day 6, when it has transited to the uterus.

Blastocyst formation occurs either during transport to the uterus or soon after. The blastocyst expands rapidly (> 300 µm by Day 7 post-ovulation) but remains spherical and the embryonic vesicle is highly mobile in the uterus. This mobility phase is essential for the maternal recognition of pregnancy (McDowell et al., 1988), which occurs between days 12-14, although the specific mechanism has not yet been identified in the horse. At around day 16, the embryonic vesicle lodges within the uterus, largely as a result of its increasing size and progesterone-induced tonic uterine contraction; however, it is still not attached to the endometrium. Trophoblastic cells invade the maternal endometrium at day 35-40, however true attachment does not occur until placentation begins on Day 45 (Samuel et al., 1974).

Another unique feature of equine embryology is the formation of an acellular glycoprotein capsule (review; Stout et al., 2005). Immediately following entry to the uterus (Day 5.5-6 post ovulation), capsule glycoproteins, produced by the trophoblast cells, coalesce under the zona pellucida to encapsulate the developing embryo. The zona pellucida is shed within a day of entering the uterus, leaving the embryonic vesicle enveloped in the capsule. The hypothesized function of the

capsule is to maintain the spherical shape and provide tensile strength whilst the vesicle is mobile and to form a barrier between endometrial and embryonic cells, preventing adhesion during the mobility phase. It has also been suggested that the negative electrostatic charge of the capsule aids in attracting the positively charged histiotroph. The capsule is lost gradually around day 21, at which point cell-cell contact with the endometrium is established.

In order to appreciate the complex requirements of an IVP system, a more detailed review of the intricate and interdependent events that occur during *in vivo* oocyte development must be appreciated.

1.3 Mammalian oocyte development

At birth, the ovaries of female mammals already contain a pool of primordial ovarian follicles containing primary oocytes. On-going follicle recruitment then ensues throughout the female's reproductive lifespan, independent of gonadotropin stimulation (Figure 1.2). Gonadotropin-independent recruitment involves the early stages of oocyte development, whereby primordial follicles (single layer of squamous granulosa cells enclosing an immature oocyte of ~30 µm diameter with no zona pellucida) develop into tertiary follicles (follicles with a many layers of cuboidal granulosa cells, an oocyte of 100-150 µm diameter with a fully developed zona pellucida and an antrum). At this point, the follicle is capable of responding to gonadotropins, and gonadotropin-dependant, cyclic recruitment of ovarian follicles occurs (Figure 1.2). FSH is the gonadotropin responsible for this cyclic recruitment. In monovulatory species (including the horse and man) a single antral follicle is generally then selected from the growing cohort of follicles and continues to grow and establish dominance. Attainment of dominance is accompanied by atresia of subordinate follicles in the recruited cohort (as described in Section 1.2.1; Figure 1.1). The dominant follicle will eventually progress to ovulate under the influence of LH (Figure 1.2).

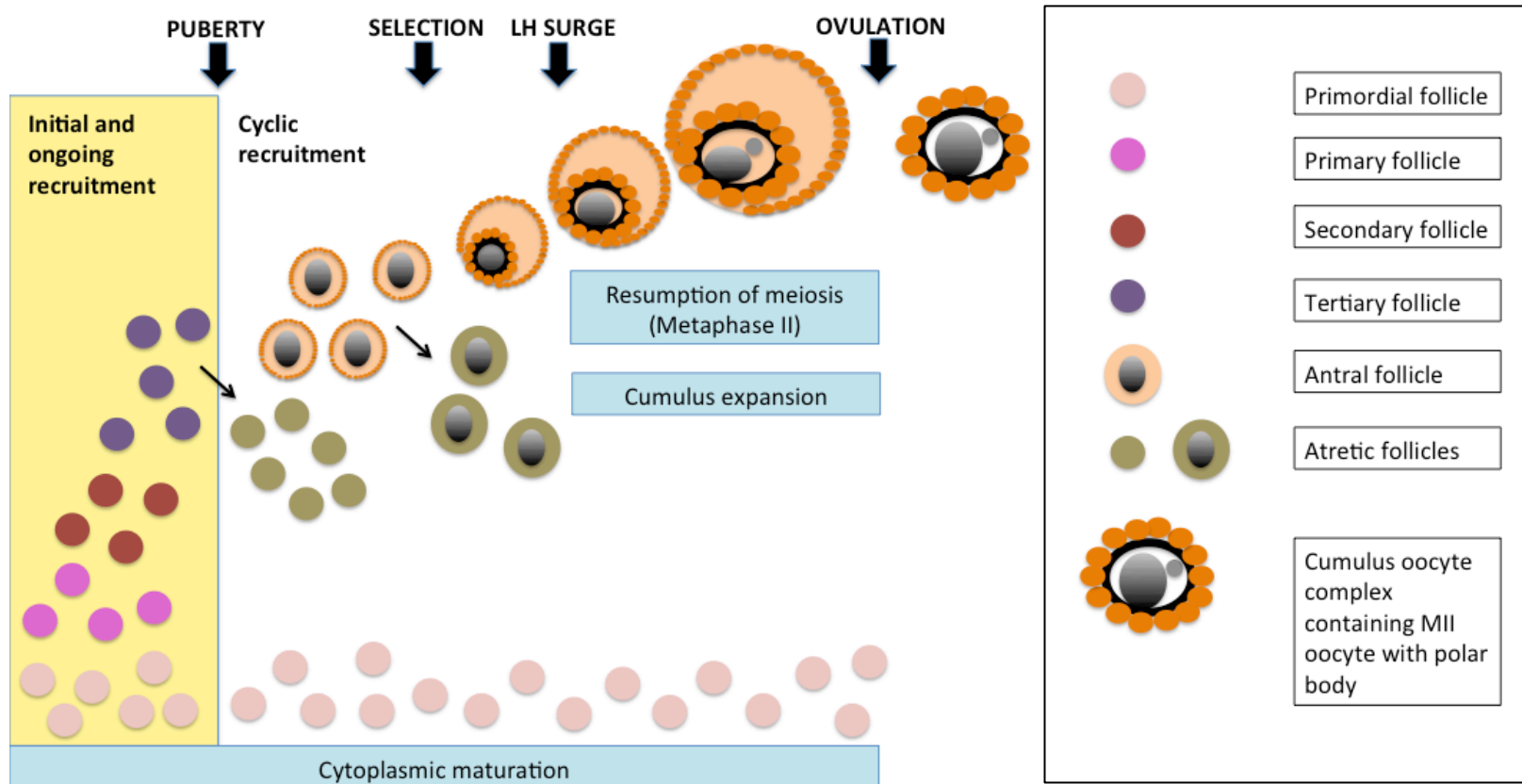


Figure 1.2: Schematic diagram of folliculogenesis and oocyte development. Initial and on-going recruitment takes place throughout the life of the female involving development from primordial to tertiary follicles which all contain an oocyte arrested in Prophase 1 of meiosis. At puberty, cyclic recruitment of antral follicles begins under the influence of follicle stimulating hormone (FSH), followed by selection, establishment of dominance and final maturation under the influence of luteinising hormone (LH) leading to ovulation of a mature Metaphase II oocyte.

1.3.1 The cumulus-oocyte complex

The cumulus cells are the granulosa cells that are in close proximity to the oocyte with the innermost layer of cells (corona radiata) having direct contact with the oocyte through transzonal processes (Figure 1.3). The cumulus cells arise from differentiation of granulosa cells at the antral stage of follicular development, when two distinct sub-populations form; mural granulosa cells and cumulus granulosa cells, both with a distinct phenotype (Eppig, 2001). Presence of cumulus cells is essential for oogenesis, folliculogenesis, meiotic and cytoplasmic maturation and energy provision to the oocyte (Figure 1.3; Ackert et al., 2001; Albertini et al., 2001; Tripathi and Kumar, 2010). While initially it was thought that the cumulus cells exclusively supported the oocyte in both a paracrine manner and through gap junctions, it was subsequently demonstrated that this communication was bi-directional, with the oocyte also providing signals to the cumulus (Figure 1.3; Eppig, 2001; Kidder and Vanderhyden, 2010). This cross talk is clearly essential to maximise the oocyte's development potential. Data from other species indicate that IVF rates are low if the cumulus cells are first removed (Vanderhyden and Armstrong, 1989; Zhang et al., 1995). Additionally, maintaining this cross-talk is key to processes essential to *in vivo* fertility such as folliculogenesis, ovulation and follicle luteinisation (Ackert et al., 2001; Eppig, 2001; Nekola and Nalbandov, 1971).

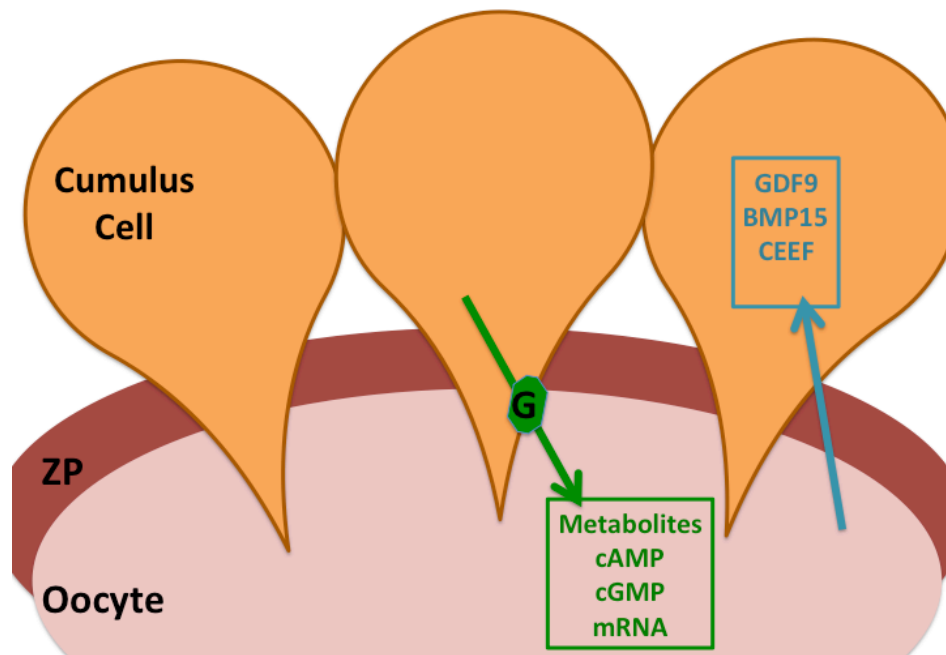


Figure 1.3: Schematic diagram illustrating bidirectional communication between the oocyte and cumulus cells. Transzonal processes project into the cytoplasm through the zona pellucida (ZP) and end in gap junctions (G) on the oocyte plasma membrane and deliver metabolites, cAMP, cGMP (Tripathi et al., 2010) and mRNA (Macaulay et al., 2016) from the cumulus cells to the oocyte. The oocyte also communicates with the cumulus cells in a paracrine regulatory manner by production of growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP 15) and cumulus expansion enabling factor (CEEf) (Eppig, 2001).

1.3.2 Meiotic maturation and competence

In female mammals, all oocytes arrest at the prophase of the first meiotic division as the primordial follicles form, around the time of birth. Meiotic arrest in the oocyte is an active process. C-type natriuretic peptide (CNP) from the mural granulosa cells causes cumulus cells to produce cyclic guanosine monophosphate (cGMP), which enters the oocyte and inhibits phosphodiesterase-3A (PDE3A) and hence cyclic adenosine monophosphate (cAMP) levels are maintained keeping the oocyte in meiotic arrest (Tripathi et al., 2010; Zhang et al., 2010). In Prophase 1, the chromosomes in the primary oocyte have already duplicated ($4n$; Note: the chromosome terminology used here refers to each sister chromatid as a separate chromosome). The oocyte nucleus is termed the “germinal vesicle” and an oocyte with an intact nucleus (thus in prophase of meiosis) is described as being in the germinal-vesicle stage. Even if removed from the suppressive environment of the follicle, oocytes are unable to progress through meiosis until the antral stage of follicular development, when the first stage of acquisition of meiotic competence occurs. At the antral follicle stage, oocytes first acquire the ability to undergo germinal vesicle breakdown (GVBD), then as the follicle develops they acquire the ability to reach metaphase II (the stage at which they are ovulated; Eppig, 2001). A metaphase II oocyte is one that has extruded its first polar body (Figure 1.2) and contains $2n$ chromosomes in the oocyte and $2n$ in the polar body). The $2n$ chromosomes in the oocyte must separate, and a second, $1n$ polar body must be extruded, in order to create a haploid cell (n). Meiosis in the female gamete (one egg [n] produced from each primary oocyte [$4n$]) is in contrast to that in the male where 4 spermatozoa (n) are produced from each primary spermatocyte ($4n$).

1.3.3 Cytoplasmic maturation

During follicular development from primordial to antral follicle, and through to the preovulatory oocyte, the cytoplasm of the oocyte also matures. Cytoplasmic maturation is essential to support fertilisation and early embryo development until embryonic genome activation (EGA; 2-8 cell stage in most species studied; Telford et al., 1990, considered to be 4-8 cell in the horse; Brinsko et al., 1995). The process of cytoplasmic maturation is relatively undefined. However, essential components of cytoplasmic maturation include formation of cortical granules and microtubules, and the elongation of mitochondria and an increase in size of the Golgi apparatus (Ferreira et al., 2009). Maternal mRNA must be transcribed and exported to the cytoplasm. While in most cells mRNA has a very short lifespan, the oocyte is unique in being able to store cytoplasmic mRNA in a stable state by shortening the polyA tail (20-40bp). The subsequent translation of mRNA is then regulated by either temporal polyadenylation (lengthening to 80-250bp) or deadenylation (Rodriguez and Farin, 2004) and timely availability of specific mRNAs is crucial for meiotic resumption, fertilization and support of the early embryo through to EGA (Mamo et al., 2011).

1.3.4 Final maturation under the influence of LH

Cumulus cells are normally tightly packed and in close communication via tight junctions during the growth phase of the follicle, forming a compact (Cp) cumulus mass. The final pre-ovulatory maturation phase of the follicle, under the influence of LH, is characterised by cumulus expansion and oocyte meiotic resumption. The tight junctions between cumulus cells detach, and the cumulus cells produce an intercellular matrix rich in hyaluronic acid, which results in a mucified matrix forming between adjacent cells, thus pushing them apart (expanded cumulus; Ex; Eppig, 1979). Simultaneously, disruption of gap junctions between the cumulus cells and the oocyte reduces input of cAMP and cGMP to the oocyte. All of these actions appear to be tied to activation of mitogen activated protein kinase (MAPK) family members and results in net reduction of intracellular cAMP in the oocyte, leading to

activation of maturation-promoting factor (MPF), GVBD and resumption of meiosis (Tripathi et al., 2010). There is also considerable cytoplasmic reorganisation of organelles like mitochondria and cortical granules, in addition to changing microtubule and actin microfilament distribution (Ferreira et al., 2009)

This sequence of events results in a secondary oocyte, which has extruded the first polar body, is arrested in the metaphase of the second meiotic division and is contained within an expanded gelatinous cumulus mass. This is the oocyte which will be ovulated and potentially fertilised.

1.4 *In vitro* embryo production in the horse

In vitro embryo production (IVP) in the horse can improve our understanding of early embryo development and has clinical applications in the salvage of valuable equine genetics (Galli et al., 2007; Hinrichs et al., 2012; Li et al., 2001). Horses are long-lived animals and at advanced ages may have accumulated a range of reproductive pathologies that preclude routine breeding success. Natural breeding and artificial insemination rely on the mare ovulating normally and having a functional tubular reproductive tract, and the fertility of the stallion's semen being adequate. *In vitro* embryo production can circumvent many reproductive pathologies, as oocytes are retrieved before ovulation and the integrity of the tubular reproductive tract is not essential for success. Additionally, minimal numbers of spermatozoa are required, for which good motility is not essential, thereby offering a mechanism to produce embryos and foals when only limited reserves of semen are available (Choi et al., 2006a).

Despite concerted efforts, standard *in vitro* fertilization (IVF) protocols have not been successfully applied to the horse in a repeatable manner (review; Leemans et al., 2016). For this reason, successful equine IVP has thus far relied on intracytoplasmic sperm injection (ICSI). The first equine pregnancy established following ICSI was reported in 1996 by Squires et al. Application of *in vitro* technologies such as ICSI first require the retrieval of oocytes, which in the live mare is most commonly performed via ultrasound-guided transvaginal aspiration (TVA; Jacobson et al., 2010). The procedure, performed under standing sedation, is well tolerated and carries little risk to health and future fertility (Velez et al., 2012). For research purposes or in the case of genetic salvage, should a mare die or be euthanised, oocytes can also be retrieved *post mortem* directly from the ovaries (Hinrichs et al., 2012). Retrieved immature equine oocytes are then matured *in vitro* before being subjected to ICSI. Briefly, the ICSI technique involves the injection of a single spermatozoon into the cytoplasm of a mature metaphase II oocyte using a micromanipulator (Figure 1.4).

Injected oocytes are cultured *in vitro* for 7-10 days until blastocyst development occurs, at which time they can be transferred transcervically to a recipient mare or cryopreserved for transfer at a later date (Hinrichs, 2010).

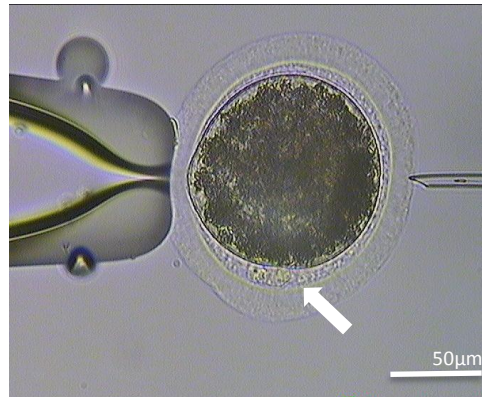


Figure 1.4: Intracytoplasmic sperm injection in an equine metaphase II oocyte. The holding pipette (left) secures the oocyte for injection by the needle containing the spermatozoa (right). The polar body can be seen at 6 o'clock (white arrow).

Development of a successful equine ICSI programme remains elusive for many. The production of viable blastocysts *in vitro* encompasses many complex and interdependent procedures and extensive financial, technical and intellectual investment. Reported blastocyst rates per injected oocyte are typically < 10 % (Alonso et al., 2014; Smits et al., 2012; Zaniboni et al., 2013). Few laboratories have published rates higher than this (Foss et al., 2013; Galli et al., 2014a; Jacobson et al., 2010; Matsukawa et al., 2007) and only two ICSI laboratories have published clinical success in a commercial programme (Galli et al., 2016; Hinrichs et al., 2014). Despite recent brief reports of good (> 20%) blastocyst rates achieved by various research groups, none have yet appeared in the peer-reviewed literature.

In other domestic species and man, controlled comparative studies to optimise the procedural steps involved in IVP, coupled with intense investigation of the *in vivo* environment, have supported the rapid improvement of IVP systems. In contrast, there has been relatively little work reported in either of these areas for the horse.

This is due in part to pressures arising from clinical demand and private industry funding, which can over-ride the necessity for strict scientific rigour and evidence-based progress. Further, legal constraints on the availability of research animals and / or limited availability of *post mortem*-derived oocytes in some geographic areas, represents another barrier to a structured approach. Given the clinical/commercial application of this technique in producing foals used for performance, and with growing knowledge of potential downstream effects on offspring as a result of an inadequate *in vitro* environment during IVP (El Hajj and Haaf, 2013; Fernandez-Gonzalez, Ramirez et al., 2007; Watkins et al., 2007), it is a priority to develop a species-tailored system for equine IVP.

1.4.1 Oocyte developmental competence

For all IVP systems, maximising the quality of the oocyte presented for fertilisation is central to success. The concept of oocyte quality, however, is complex and ill defined; the only true measure being the ability of the oocyte to produce a viable embryo, establish an on-going pregnancy, and result in a full term healthy offspring. Thus, the term often used for oocyte quality, which reflects adequate oocyte cytoplasmic maturation, is 'oocyte developmental competence'. In a research setting, studies rarely have the scope or funding to allow the production of offspring, therefore the developmental potential of the oocytes must often be extrapolated and predicted from either fertilisation, cleavage or blastocyst production data.

In many species, including humans, oocyte developmental competence is maximised by stimulating the donor female to produce multiple *in vivo*-matured oocytes through follicle super stimulation and administration of an LH-like stimulus to induce final follicle maturation. However, attempts at superovulation have largely been unsuccessful in the horse, due to the lack of availability of equine FSH, the variable and relatively poor response (average of 3 follicles ovulating; Meyers-Brown et al., 2010; Niswender et al., 2003) and the large size of the pre-ovulatory follicles, making manual manipulation of the ovary technically difficult if follicle

aspiration is performed (Maclellan et al., 2002). For this reason, equine IVP relies on aspiration of either the one dominant preovulatory follicle (Carnevale, et. al., 2013; Maclellan et al., 2010) or of immature follicles to recover germinal vesicle-stage oocytes, which must subsequently undergo IVM. In the single study reporting rates achieved with both of these techniques, the blastocyst rate per aspiration obtained when all immature follicles were aspirated (one blastocyst per aspiration) was greater than that obtained when the dominant stimulated preovulatory follicle alone was aspirated (0.33 blastocysts per aspiration; Jacobson et al., 2010).

Thus when producing equine embryos or foals via *in vitro* methods, both understanding the factors related to oocyte developmental potential intrinsic to the immature oocyte before collection, and maximising the developmental competence of the immature oocytes during the IVP process leading to ICSI, are central to success.

1.4.2 Intrinsic factors affecting oocyte developmental competence

Mare factors: Age, breed, reproductive disease and fitness

The phenotype of the donor female is known to affect oocyte developmental potential in many species, with age being the most relevant factor in the human (Navot et al., 1991). In the mare, several authors have investigated mare factors affecting clinical outcomes of IVP, often reporting conflicting results. Interestingly, while a negative correlation has been demonstrated between increased age in donor mares and oocyte recovery rate (Claes et al., 2016; Hinrichs et al., 2014), mare age did not impact either maturation or blastocyst rates¹ in either of the studies.

Mare athletic fitness (mare actively participating in a performance discipline), mare breed and reproductive disease have all been negatively correlated with outcome. Colleoni et al., (2007) reported a decreased blastocyst rate for mares engaged in

¹ Blastocyst and cleavage rates are given as number of blastocysts/cleaved embryos obtained per number of metaphase II oocytes subjected to ICSI unless otherwise stated.

athletic performance activities vs. those that were not (3.9% vs. 15.25% respectively) while Claes et al., (2016) reported mares with subfertility related to uterine abnormalities to be 13 times less likely to produce and embryo than fertile mares when evaluating blastocyst production per aspiration session. Galli et al., (2014b), reported a decrease in maturation, cleavage and blastocyst rates in Arabians compared to both Warmbloods and Quarter horses. However, both athletic performance and reproductive disease have been found to be independent of outcome in other studies (athletic fitness; Claes et al., 2016; reproductive disease; Colleoni et al., 2007)

Season and cycle stage

It has been reported that while season (breeding vs. non-breeding season of the mare) exerts an effect on follicle diameter (increased diameter during breeding season; Hinrichs and Schmidt, 2000), follicle number (increased number during non-breeding season; Choi et al., 2016b) and oocyte recovery rate (higher recovery rate during spring transition than fall; Claes et al., 2016) it does not impact maturation, cleavage or blastocyst rates of recovered oocytes (Choi et al., 2016b; Colleoni, 2007;).

The stage of the mare's oestrus cycle has also been demonstrated to affect oocyte characteristics. Two successive studies demonstrated that aspiration at the end of the follicular phase (34 hours after induction of ovulation with Crude Equine Gonadotropins; CEG) resulted in the greatest IVM rates compared with aspiration during the luteal phase (34 hours after largest follicle reached 18mm in the presence of a corpus luteum; Goudet et al., 1997 and 1998). For these reasons, oocytes collected during the luteal phase may not perform well in an IVP system, due to the increased proportion of COCs with compact cumulus and higher prevalence of juvenile chromatin compared to collection at the end of the follicular wave. However, none of these studies evaluated developmental potential, therefore the optimal time to collect immature oocytes during the oestrous cycle is still unknown.

Cumulus classification

Across species, the morphology of the cumulus of the recovered oocytes is considered an important factor in grading oocytes as a predictor of meiotic and developmental competence. In the horse, this is complicated by the discrepancy among laboratories as to classification of cumulus morphology, making comparisons of results among laboratories difficult. As described in Section 1.3.4, cumulus and mural granulosa cells are tightly arranged in close proximity (compact; Cp) during the growth stages of the follicle and become expanded (Ex) during the final maturation stage under the influence of LH. Follicular atresia is another reason that cumulus and granulosa cells become expanded in appearance, in this instance due to apoptosis and loss of cell-cell communication (Hinrichs and Williams, 1997). When performing TVA for clinical purposes in the live mare, all visible follicles (≥ 5 mm diameter on transrectal ultrasound-generated images) are aspirated and immature oocytes are typically recovered with the majority of the cumulus absent due to the close attachment of the cumulus to the follicle wall (Hawley et al., 1995) and present as corona radiata-only (Figure 1.5A). By contrast, immature oocytes recovered *post-mortem*, by scraping of the follicular walls with a bone curette (Hinrichs and DiGiorgio, 1991), are generally retrieved with a greater proportion of the granulosa cells intact and can be accurately classified (Figure 1.5B-D). The scraping technique maximises recovery rates (up to 85% vs. 40-60% for aspiration; Dell'Aquila et al., 2001). This thesis centres on oocytes obtained *post-mortem*. In accordance with this, the following descriptions focus on the collection of oocytes from *post-mortem* ovaries.

In the horse, the viable follicle prevents even the acquisition of meiotic competence by the enclosed oocyte until the follicle is > 20 mm diameter; (Hinrichs and Schmidt, 2000), which for the follicle destined to ovulate is approximately a week before ovulation. However, oocytes within atretic follicles rapidly acquire meiotic competence (Hinrichs and Williams, 1997), presumably due to breakdown of granulosa/oocyte communications and reduction in cAMP levels (Section 1.3.4). When placed in culture *in vitro*, those oocytes that have acquired meiotic competence spontaneously mature when removed from the follicle. By contrast,

those that have not acquired meiotic competence will fail to mature when placed in culture and instead degenerate. These findings support the fact that in the horse, the highest maturation rates are from oocytes derived from either large (> 20 mm diameter) viable growing follicles (with compact cumulus, Cp) or from atretic follicles of any size > 5 mm. (with expanded cumulus Ex; Figure 1.5; Hinrichs and Schmidt, 2000; Hinrichs and Williams, 1997)

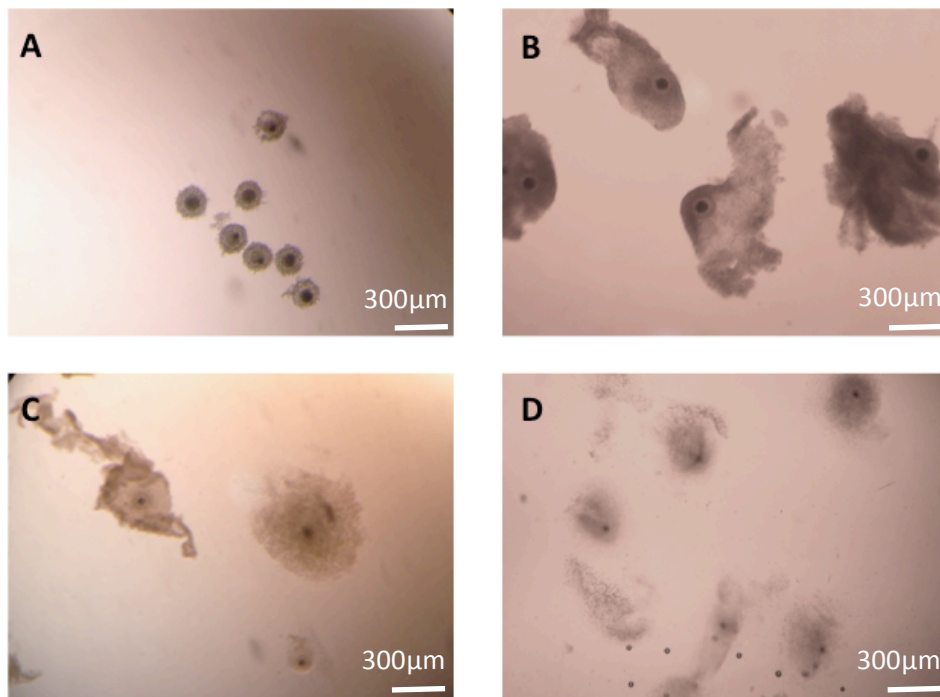


Figure 1.5: Bright field photomicrographs of immature cumulus oocyte complexes (COCs). A: Corona radiata-only COCs obtained via transvaginal aspiration (TVA). B: Compact intact-cumulus COCs (Cp) obtained via follicular scraping of post-mortem ovaries. C and D: Expanded intact-cumulus COCs (Ex) obtained via follicular scraping of post-mortem ovaries.

Unfortunately, of antral follicles found in horse ovaries, few (< 5%) follicles are >20 mm diameter (Hinrichs and Schmidt, 2000). Thus, the majority of the meiotically-competent oocytes recovered are those from follicles in atresia.

Overall, if cumulus morphology is rigorously evaluated, it is a useful predictor of meiotic competency. If all complexes showing any sign of cumulus expansion are classified as Ex, Ex oocytes have a higher maturation rate than do Cp oocytes (> 60% vs. 20-30%; Hinrichs et al., 1993). Cumulus classification (Ex or Cp) did not have any effect on blastocyst development rates after ICSI (Hinrichs et al., 2005); however, little is known about the further developmental competence of these oocyte types.

1.4.3 Factors related to the IVP process

In addition to the intrinsic factors discussed above, the elements of the *IVP* process preceding ICSI (transport, handling, and IVM) also have the potential to affect developmental competence. These factors are discussed below as they relate to immature oocytes retrieved from ovaries *post mortem*.

Ovary transport factors: Temperature and duration of transport

Due to the sparsity of horse abattoirs in many geographic areas, slaughter generally occurs at a location remote to the laboratory where the *IVP* takes place. As such, transport factors must be considered for each individual laboratory, in order to maximise viability of the oocytes on arrival at the laboratory. The temperature and duration of ovarian transport are two major factors that can affect success.

While some studies have found no difference in maturation rates according to transport time (1.5-4 vs. 6-8 hours; [Guignot et al., 1999]), Hinrichs et al., (2005) reported that a delay of < 1 hour between slaughter and onset of *IVM* resulted in greater oocyte maturation rates than following a 5-9 hour delay. Conversely, the delay in transport time was associated with an increased blastocyst development rate (29% vs. 13%).

The effect of ovarian transport time on blastocyst development has been evaluated in other studies, however the confounder of temperature is often problematic. Ribeiro et al., (2008) found that holding ovaries for 7 hours (at either 24°C, or at a temperature slowly descending from 35 °C) had no impact on maturation rate when

compared to ovaries slowly cooled to 27° C over 4-6 hours but did decrease blastocyst development (16.2-18.2% vs. 34%). However, a total holding time of 20 hours decreased both oocyte maturation (29-38% vs. 57%) and blastocyst rate (12-14.8 vs. 34%) compared to those cooled to 27° C over 4-6 hours. Another study reported a blastocyst rate of up to 25.5% after 24 hours of ovary storage at 15-20°C (Matsukawa et al., 2007). When pregnancy rates after surgical transfer of IVM oocytes to the oviducts of recipient mares were used as a metric, similar outcomes were achieved for oocytes derived from ovaries transported at either 12 or 22 °C for 18-24 hours (15% vs. 18% respectively; Preis et al., 2004). Clinically, healthy foals have been produced from mares that died up to 9 hours before oocyte collection (Hinrichs et al., 2012).

In summary, data suggest that ovarian transport of up to 5-9 hours at ambient temperature, thereby allowing a slow cooling curve, allows for the greatest blastocyst rates. However, these studies are by no means exhaustive, therefore the optimal conditions are still unknown.

In vitro maturation

The first successful equine IVM was reported by Fulka and Okolski, (1981) and the first embryo from an IVM oocyte in 1989 by Zhang *et al.*, after oviductal transfer into an inseminated mare and uterine flushing 7 days later. Today, the majority of equine laboratories engaged in IVM use commercially available cell culture media with or without modifications, and protocols vary widely. The two most commonly used media for equine IVM are TCM-199, originally designed for chick embryo fibroblasts and first reported by Zhang et al., (1989) and DMEM-F12, also a complete tissue culture medium, first described for use in equine IVM by Galli *et al.*, (2007).

Logic suggests that when considering the optimal media for IVM, it should be the one that most resembles the follicular environment in which maturation occurs *in vivo*. However, interpretation of data on the composition of follicular fluid aspirates is problematic. Given its dynamic nature, it is difficult to ascertain whether the

concentrations of components measured in follicular fluid are those that should be provided, those that are surplus to requirements, or those that represent metabolic by-products of granulosa cell metabolism.

In contrast to other species, there has been relatively little work reported on the composition of equine follicular fluid, with the exception of quantitative studies characterising steroid hormones. Collins et al., (1997) described both follicular fluid and serum at different time points in the oestrous cycle. They found that concentrations of glucose, the major carbohydrate substrate, were decreased in follicular fluid compared to serum, and that glucose concentrations further decreased as ovulation approached ($4.72 \pm 0.29\text{mM}$ 24 hours after dominant follicle selection [20-25mm] vs. $3.24 \pm 0.09\text{mM}$ 33 hours after induction of ovulation with CEG). A brief report, by Gerard et al., (2000) also stated that glucose tended to decrease as ovulation approached and this was confirmed by the same group in 2002 using qualitative nuclear magnetic resonance (NMR) spectroscopy (Gérard et al., 2002). It is tempting to speculate this is due to increased consumption of glucose by the COC and mural granulosa cells.

Follicular fluid was first investigated as a medium for maturation *in situ*. Maturation was attempted via intra-follicular transfer of immature oocytes into the preovulatory follicle (Hinrichs and DiGiorgio, 1991). These authors showed that the preovulatory follicle was capable of inducing cumulus expansion in immature oocytes after 24 hours, and also that the follicular environment was capable of supporting complete nuclear and cytoplasmic maturation of the transferred oocytes. In this study, the number of embryos recovered on days 7-11 exceeded the number of ovulations in 4/15 mares that had intra-follicular transfer performed. Goudet et al., (1997) also performed intra-follicular transfer and compared the nuclear maturation rates of oocytes transferred to and recovered from the follicle, to those of *IVM* oocytes. They found increased cumulus expansion in the transferred oocytes but no difference in maturation rates, however 50% of transferred oocytes were lost.

Following on from these findings, follicular fluid received attention as both a complete *IVM* media and as a supplement to *IVM* media. Bogh et al., (2002) performed *IVM* in pure follicular fluid and found that while it did not increase maturation rates it supported cumulus expansion equal to that of M199 supplemented with gonadotropins, but only when collected 35 hours after CEG stimulation. Dell'Aquila *et al.*, (1997) found that as a 20% supplement, it did not increase maturation or fertilisation rates after conventional IVF, but it did increase normal fertilisation post ICSI. Unfortunately, the use of follicular fluid, either pure or as a supplement, has not been experimentally re-evaluated since blastocyst production has been possible via ICSI but its use has been reported (supplemented at 9% of total volume and blastocyst rates up to 37%; Foss et al., 2013).

In considering the commercial media used in equine *IVM* and more specifically their glucose concentrations, M199 (at 5.6 mM) supplies glucose near the range found in the follicular fluid and in equine serum (4.9- 6.mM), and DMEM/F12 (at 17 mM), supplies supraphysiological concentrations. However, both systems have produced viable embryos and healthy offspring after ICSI and embryo culture (Galli et al., 2007; Hinrichs et al., 2014; Smits et al., 2010). Galli *et al.*, (2007) reported that DMEM/F12 was superior to M199-based *IVM* media when considering subsequent blastocyst development (26.4% [37/140] for DMEM-F12 vs. 12% [23/191] for M199). Other laboratories have found the opposite (K. Hinrichs, personal communication). Notably, the report of Galli et al. (2007) has encouraged many new laboratories to use DMEM/F12 as a base for maturation media; however, there is a significant variation in the composition of DMEM/F12-based *IVM* media between groups. This variation is in part a result of differences in composition between suppliers and is compounded by the inclusion of often undisclosed laboratory-specific modifications. Media additives are often included at varying concentrations, such as pyruvate, somatotropin, FSH, LH, insulin-transferrin-selenium (ITS), epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) (Foss et al., 2013; Galli et al., 2002). The evidence-base for these modifications is rarely presented. The few that have been critically evaluated with respect to meiotic and developmental competence are discussed below.

Equine IVM is generally performed in the presence of either mare serum or foetal bovine serum (FBS). Zhang et al., (1989) reported that the inclusion of FBS and gonadotropins (FSH/ LH/ somatotropin) increased the rate of cumulus expansion compared to mare serum (89 vs. 13%) but did not change the maturation rate (63-64%). Willis et al., (1991) reported similar findings for addition of FSH to media containing mare serum (increased cumulus expansion but no change in maturation rate). Interestingly, in that study, when FBS was used instead of mare serum, it independently increased the rate of cumulus expansion (80 to 100%). More recently, Dell'Aquila et al., (2004) examined the effect of FSH in a semi-defined medium containing bovine serum albumin (BSA) instead of serum and found that addition of FSH (1 µg/ml) increased rate of cumulus expansion but not as much as did addition of 20% FBS. These authors also reported no effect on maturation rate.

Growth factors have been studied as potential additives to equine IVM media due to their regulatory role in ovarian function and positive impact on IVM outcomes in other species. Both EGF and IGF1 have been shown to increase maturation rates, but only when used in the absence of serum (Lorenzo et al., 2002 and Carneiro et al., 2001 respectively). Carneiro et al. (2001) while not observing a positive effect of IGF1 on maturation rate (in the presence of serum and gonadotropins) did report an increase in cleavage rate after parthenogenetic activation, potentially demonstrating a positive effect on cytoplasmic maturation. While some groups include growth factors routinely (Lagutina et al., 2005), there are no studies demonstrating a positive effect on embryo development in an ICSI system.

Interleukins (paracrine factors involved in ovulation) specifically interleukin 1 β (IL1 β) have also received attention. Martoriati et al., (2002) were the first to demonstrate that equine oocytes and cumulus cells expressed IL1 β and that expression was lower in IVM oocytes compared to immature or *in vivo*-matured oocytes. Addition of IL1 β to IVM media however, decreased maturation rates (Martoriati et al., 2003, 2002); this was confirmed in a later study by Caillaud et al., (2008).

Compounds to protect the oocyte from oxidative damage have also been investigated, with the aim being to increase intracellular glutathione levels, shown to be beneficial in other species. However, neither cystamine (Deleuze et al., 2010; Luciano et al., 2006) nor β -mercaptoethanol (Merlo et al., 2016) had a positive effect on maturation rate (Luciano et al., 2006), cleavage rate after ICSI (Merlo et al., 2016) or embryo development rate after oocyte transfer (Deleuze et al., 2010).

Of interest, an M199-based *IVM* medium containing only 10% FBS and 5 mU/ml of FSH has produced the highest reported blastocyst rates ($\geq 40\%$ in numerous reports; Hinrichs et al., 2005; Jacobson et al., 2010; Ribeiro et al., 2008). There is still clearly insufficient information from controlled comparative studies as to what constitutes an ideal equine *IVM* medium in terms of both maximising embryo development and physiological suitability for the horse.

1.5 A metabolic approach to define optimal requirements for oocyte maturation².

Studying energy metabolism has been used as an approach to define and maximise oocyte developmental potential during IVM. Improving our understanding of the basic physiology and metabolism of the oocyte informs the design of a species-specific maturation medium tailored to the substrates required by the relevant, active pathways. A further driver to study metabolism is the desire to identify biomarkers of viability and to subsequently design non-invasive methods for determination of individual oocyte developmental competence. Defining the 'normal' resting state of oocyte metabolism also allows the identification and description of any metabolic perturbations when they occur.

While in this thesis, metabolism, in its function to generate metabolic energy in the form of adenosine triphosphate (ATP) is focused on, the term 'metabolism' of course encompasses a significantly wider range of functions and this is particularly true for the oocyte and developing embryo. For example, oocyte metabolism includes DNA synthesis, protein synthesis, cell signalling mechanisms (Manser and Houghton, 2006), generation of Reactive Oxygen Species (ROS) (Agarwal et al., 2005), redox balance (Sutton-McDowall et al., 2010) and gene expression in terms of establishment of epigenetic marks such as methylation and acetylation (DeBerardinis and Thompson, 2012).

For the horse, essentially all of the above aspects of oocyte and COC metabolism remain to be defined. The first step, as in all species studied to date, is to characterise the mechanisms and substrates involved in ATP production by the COC during culture. Detailed consideration of these processes in eukaryotic cells is central to improving our understanding of metabolic function in the oocyte and COC.

² Elements of the following section have been published. (Lewis and Sturmey 2015; See Appendix 1)

1.5.1 The generation of ATP

In all cells, ATP is generated as a result of two processes; glycolysis and/or oxidative phosphorylation (OXPHOS). At the intracellular level, glucose is the substrate that enters the glycolytic pathway. The glucose molecule is broken down to two pyruvate molecules (Figure 1.6), which in turn can either be converted to lactate, or to acetyl-CoA, and enter the tricarboxylic acid (TCA) cycle. Fatty acids and amino acids can also be converted to acetyl-CoA and enter the TCA cycle. The TCA cycle generates reduced nicotinamide adenine dinucleotide (NADH) molecules (Figure 1.7), which act as electron carriers in the electron transport chain during oxidative phosphorylation, with the end result being ATP production (Figure 1.8).

Glycolysis

Glycolysis is a cytosolic process by which glucose ($C_6H_{12}O_6$) is metabolised to pyruvate ($C_3H_4O_3$) to provide a net of 2 molecules of ATP per glucose molecule (Figure 1.6). Briefly, glucose first enters the cell via glucose transporters and is then immediately phosphorylated by the enzyme hexokinase (HK) into glucose-6-phosphate. The rate-limiting step of glycolysis is the reaction in which fructose 6-biphopshate is produced from fructose 6-phosphate by the enzyme phosphofructokinase (PFK). A series of reactions then proceed in parallel, resulting in the production of two pyruvate molecules and a net of two molecules of ATP.

Pyruvate can either be converted directly to lactate or to Acetyl-CoA. Conversion to lactate, as in anaerobic glycolysis, occurs when oxygen supply is limited or there is an urgent ATP requirement. Preferentially metabolising glucose via glycolysis in the presence of plentiful oxygen is known as the 'Warburg effect' and is characteristic of cancerous tumour cells (Warburg, 1925). Conversion of pyruvate to Acetyl-CoA allows for transport to the mitochondria for further ATP production via the TCA cycle and oxidative phosphorylation. The rate of glycolysis is controlled by two main mechanisms. The first is ATP abundance, whereby high intracellular concentrations can inhibit PFK through negative feedback causing glycolysis to proceed more slowly. The second is the $NAD^+ : NADH$ ratio. The conversion of glyceraldehyde 3-

phosphate to 1,3-biphosphoglycerate has an absolute requirement for NAD^+ (Berg et al., 2002). As the production of lactate from pyruvate yields NAD^+ , the pyruvate: lactate ratio is tightly controlled.

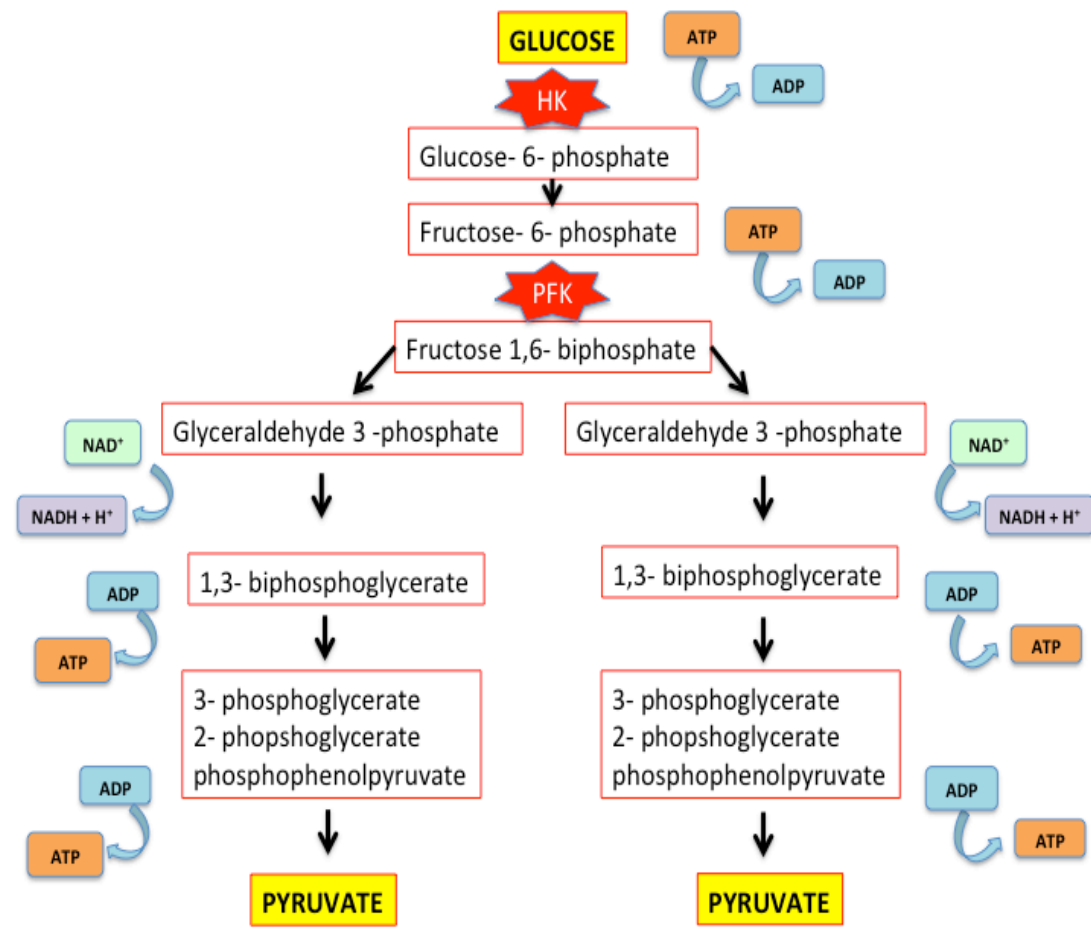


Figure 1.6: Schematic diagram illustrating the sequential reactions involved in glycolysis by which two pyruvate molecules are generated from one glucose molecule. Abbreviations are as follows; HK = hexokinase enzyme, PFK = Phosphofructokinase enzyme, ATP = Adenosine triphosphate, ADP = adenosine diphosphate, NAD = nicotinamide adenine dinucleotide, NADH = reduced NAD.

Tricarboxylic acid cycle

The TCA cycle occurs within the matrix of the mitochondria and is the series of chemical reactions by which Flavin adenine dinucleotide (FADH_2) and NADH are produced from a series of oxidation reactions beginning with Acetyl- CoA (Figure 1.7). Acetyl-CoA can be derived from either pyruvate as described above, fatty acids (through β -oxidation) or amino acids (through oxidative deamination) (Berg et al., 2002)

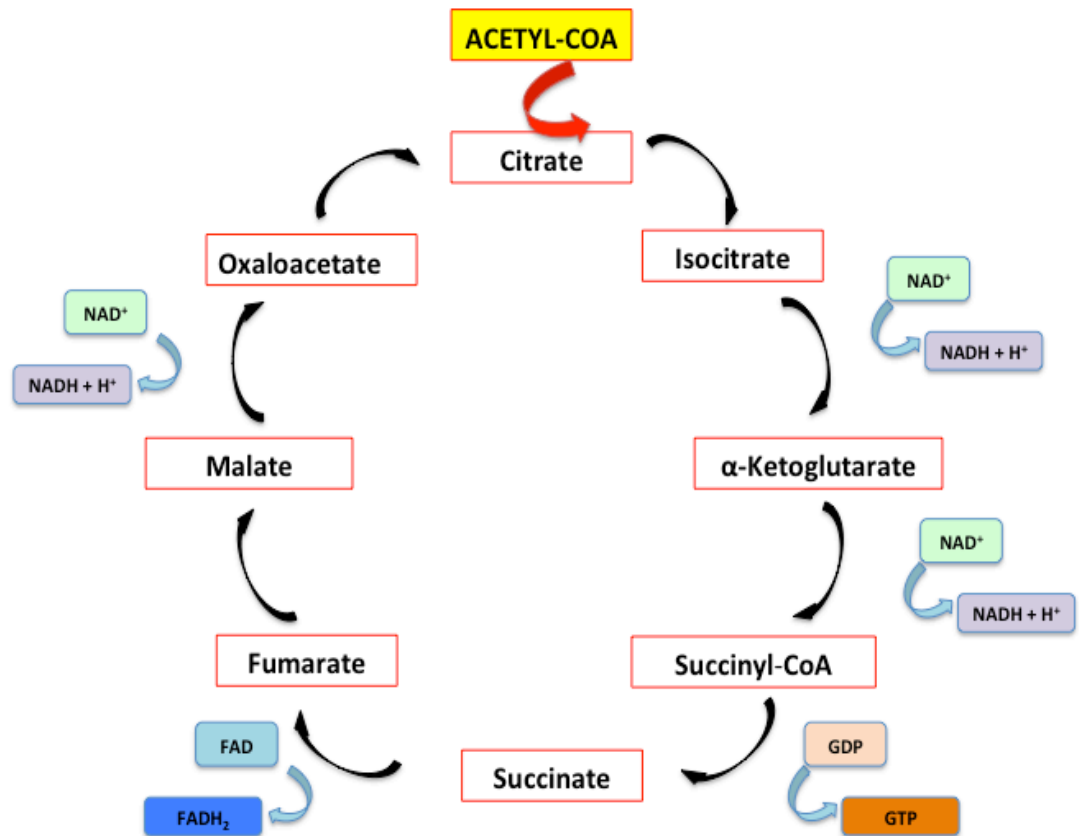


Figure 1.7: Schematic diagram illustrating the sequential reactions involved in the tricarboxylic acid (TCA) cycle by which a series of oxidation reactions beginning with Acetyl- CoA produces NADH and FADH_2 . Abbreviations are as follows; ATP = Adenosine triphosphate, ADP = adenosine diphosphate, NAD = nicotinamide adenine dinucleotide, NADH = reduced NAD, FAD = Flavin adenine dinucleotide, FADH_2 = reduced form FAD, GDP = Guanosine diphosphate, GTP = guanosine triphosphate.

Oxidative Phosphorylation

Oxidative phosphorylation (OXPHOS) involves the transfer of electrons in series of redox reactions through four successive proteins (the electron transport chain) located on the inner mitochondrial membrane. Hydrogen ions are simultaneously pumped into the intermembrane space, creating a proton gradient. This proton gradient is ultimately used to create ATP from Adenosine diphosphate (ADP) due to the passive flow of protons (H^+) through the enzyme ATP synthase (Figure 1.8). Up to fifteen ATP molecules can be generated from each Acetyl- CoA molecule that enters the TCA cycle (a theoretical maximum of 30 from each initial glucose molecule). As oxygen is the terminal electron acceptor, combining with hydrogen to form water, oxygen consumption rate (OCR) can be used as an overall measure of OXPHOS (Mookerjee et al., 2017). However, there are several reasons why this is not the case in all cells for all species. These will be discussed below with respect to mitochondrial function.

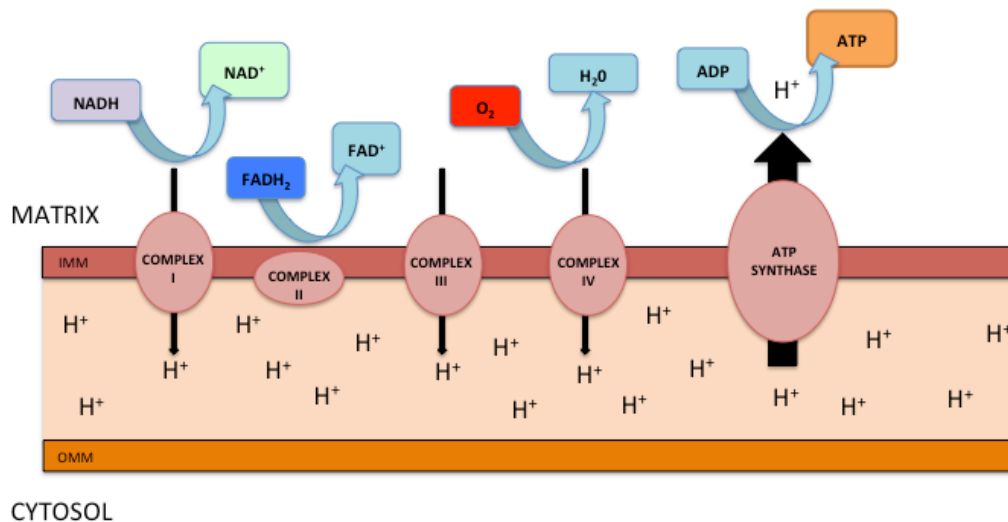


Figure 1.8: Schematic diagram illustrating the electron transport chain during oxidative phosphorylation. Abbreviations are as follows; ATP = Adenosine triphosphate, ADP = adenosine diphosphate, NAD = nicotinamide adenine dinucleotide, FADH₂ = Flavin adenine dinucleotide, NADH = reduced NAD, IMM = inner mitochondrial membrane, OMM = outer mitochondrial membrane.

1.5.2 Mitochondrial function

Oxidative phosphorylation in the mitochondria is responsible for the majority of cellular oxygen consumption. However some of the oxygen consumption in a cell can be attributed to production of reactive oxygen species (ROS) and to oxygen-consuming enzymes such as NADPH oxidase, xanthine oxidase and squalene mono-oxygenase. Of the oxygen consumption attributable to OXPHOS, 60-80% is coupled to ATP production in the majority of mammalian cells studied (Divakaruni and Brand, 2011). Oxygen consumed but not coupled to ATP production is either non-mitochondrial, as noted above, or is the result of proton leakage across the mitochondrial membrane, bypassing ATP synthase. Mitochondrial function and integrity are crucial to the developmental potential of the oocyte. All mitochondria in the embryo are maternal in origin and there is no replication possible until the blastocyst stage in the horse (Hendriks et al., 2018), therefore damage incurred during oocyte maturation will impair oocyte competency, and will transfer to the next generation (Smeets, 2013).

Reactive oxygen species

Under normal conditions, electrons flow through the electron transport chain until the final step of water formation from oxygen. However, up to 2% of electrons may leak from complex I and III and result in single electron transfer to oxygen which produces superoxide instead of water (Starkov, 2008). Superoxide is ultimately converted to hydrogen peroxide by the enzyme superoxide dismutase, in which form it can leave the mitochondria. While ROS have a physiological role in normal cell function (Agarwal et al., 2005), they are considered highly reactive when produced in excess of a cell's antioxidant defence capacity. When this imbalance occurs, the cell is said to be in oxidative stress and the ROS can cause significant damage to mitochondrial and nuclear DNA, organelles and phospholipid membranes (Agarwal et al., 2005).

The level of ROS production is dependent upon environmental oxygen (greater ROS production at higher O₂ tension; Legge and Sellens, 1991) but can also be self-regulated by ATP demand/production. If ATP demand is low, the proton gradient (membrane potential) is high, which can result in excess electron leakage and thus increased ROS production. However, when there is active ATP production in situations of high demand, the membrane potential is reduced therefore reducing electron leakage and ROS production (Starkov, 2008).

Proton Leak

A certain degree of proton leak, defined as hydrogen ions 'leaking' back to into the matrix through the inner mitochondrial membrane but not through ATP synthase, is physiological in most cell types. In some organisms such as the rat, it accounts for up to 25% of basal metabolic rate (Rolfe et al., 1999). Brand, (2000) introduced the hypothesis of "uncoupling to survive", by which proton leak presents a method of regulating the mitochondrial membrane potential and therefore regulating ROS production. As proton leak can also be induced or increased via uncoupling proteins, the extent of leakage is under transcriptional control (Divakaruni and Brand, 2011).

1.5.3 Studying metabolism

The measurement of oocyte and COC energy metabolism is technically challenging. Data available inform us of substrate depletion and appearance in a given milieu. *In vitro*, this milieu is constrained by the addition of a limited number of substrates at static levels; supply and ratio of substrates varies only in response to an oocyte/COC's own activity. This is in stark contrast to the situation *in vivo*, which is dynamic and responsive (Leese *et al.*, 2008) and still largely unknown for most species.

Given the heterogeneity in developmental potential among oocytes recovered from immature follicles, measures pertaining to single oocytes and/or COCs are key and thus highly sensitive assays are needed. Both the use of radiolabelled substrates (Rieger et al., 1992) and enzyme-linked fluorescence assays to detect the

appearance and disappearance of a substrate from culture media have been described (Leese and Barton, 1984; Guerif *et al.*, 2013). The relative metabolic quiescence of single oocytes and COCs means that 'analysis media' is often used in order to permit detection of changes in substrate concentration (Hardy *et al.*, 1989; Sturmey and Leese, 2003). This 'analysis media' is often different from the *in vitro* culture media known to support development for most species, which, in turn differs vastly from the *in vivo* environment. Of course, it also must be realized that there are many complex pathways involved, and influx and efflux leads us to make what are essentially informed conclusions about what occurs in the interim. Despite these limitations, these assays have greatly advanced our knowledge of metabolic pathways and have yielded repeatable results across different laboratories. New promising studies using nuclear NMR metabolomic technology, in which substrate flux can be measured *in situ*, have been recently described (Krisher *et al.*, 2015), however the subsequent interpretation and analysis of the complex data acquired presents further challenges.

Inferences about the extent of oxidative metabolism are usually derived from measuring oxygen consumption. Methods vary, the most widely used being pyrene fluorescence (Harris *et al.*, 2009; Houghton *et al.*, 1996), nanorespirometry (Lopes *et al.*, 2010) and scanning electronmicroscopy (Sugimura *et al.*, 2012). Again while allowing accurate measurement of oxygen depletion, the methods represent a significant 'alien' environment for the oocyte or COC.

Studies involving metabolic inhibitors and enzymatic co-factors have also added to our knowledge of oocyte metabolism and in some cases provided the initial proof of certain pathways existing and either being essential or non-essential for development. Among these is fatty acid β -oxidation, the breakdown of fatty acids to yield ATP. Etomoxir and methyl palmoxirate, both inhibitors of carnitine palmitoyltransferase-1 (CPT1) the rate-limiting step in β -oxidation, have successfully been used during IVM to demonstrate the critical role of this pathway in optimal development. Specifically, blastocyst development in the mouse and cow is

decreased while nuclear maturation in the mouse, pig and cow is inhibited (Dunning et al., 2010; Ferguson and Leese, 2006; Paczkowski et al., 2013).

Another approach often used to explore COC metabolism is to evaluate relative gene expression. This can involve evaluating metabolic enzyme genes, such as PFK, in order to infer activity of different pathways, or evaluating genes involved in defense mechanisms such as glutathione peroxidases, which are upregulated in states of oxidative stress (Van Hoeck et al., 2013). Gene expression of developmental markers in blastocysts has also been used to determine viability and/or impact of metabolic perturbations during *in vitro* culture (Choi et al., 2015).

When we are measuring oocyte and/or COC metabolism are we just measuring a stress response? This question must be considered given the extremely adaptable nature of oocytes of all species. Metabolism is necessarily dynamic, enabling rapid changes in response to maintain development. However, such dynamism means that the metabolic profile can change quickly in response to a change in external environment, shown clearly in mouse and rat embryos, where metabolic perturbations such as elevated glycolysis and decreased pyruvate oxidation occurred after only three hours of *in vitro* culture in flushed *in vivo* blastocysts (Lane and Gardner, 1998). Both the presence and relative quantities of metabolic substrates in the environment in which experiments are conducted will significantly affect the results. In addition to the oocyte adapting to its environment, the culture environment itself is not static. Depletion of substrates and buildup and excretion of metabolites such as lactate and amino acids will change the local environment. Spontaneous deamination of certain amino acids will occur at 37°C, resulting in ammonium accumulation (Gardner and Lane, 1993). Lactate build-up in the media may overwhelm the pH-buffering system of the media. Depletion of specific energy substrates can promote the use of alternative ATP-generating pathways (Kane, 1987). It is thus essential to consider that studies on embryo metabolism only provide a 'snapshot' of physiology in a given set of conditions. Whilst such data is of fundamental importance, care must be taken when extrapolating and comparing such information.

Choosing a target- oocyte or COC?

In the case of the oocyte, it must first be decided whether to study the metabolism of the oocyte alone or that of the COC, as it co-exists *in vivo*. While studying the COC as a whole creates difficulties in analysing and assigning metabolic data to one of the two cell types, it is substantially more physiological to study them together due to the interdependent nature in which they co-exist as described in Section 1.3.3. In this regard, a brief summary of COC metabolism follows. Individual aspects will be covered in more detail in subsequent chapters.

1.5.4. Metabolism of the cumulus-oocyte complex

Metabolic regulation between the oocyte and cumulus is a finely controlled partnership. This relationship was first highlighted by Biggers et al., (1967) who demonstrated that the oocyte was not capable of metabolising glucose but rather the cumulus cells provided the oocyte with the pyruvate for subsequent ATP production. It was later discovered that the reasons for this were the relatively low expression of glucose transporters and absence of PFK in the oocyte (Cetica et al., 2002). The now-accepted mechanism (summarised in Figure 1.9) is that the cumulus cells metabolise glucose via glycolysis for both their own ATP production and for providing pyruvate to the oocyte (Harris et al., 2009) and the oocyte subsequently utilises this pyruvate for ATP production through the TCA cycle and OXPHOS (Steeves and Gardner, 1999).

Intracellular glucose in the COC can be used in pathways other than those generating ATP. These are the pentose phosphate pathway (PPP), hexosamine biosynthesis pathway (HBP) and the polyol pathway (Figure 1.9). The PPP, while representing a small proportion of glucose consumption, is essential to oocyte maturation (Downs and Utecht, 1999; Xie et al., 2016). It provides both NADPH to maintain redox balance and phosphoribosyl pyrophosphate (PRPP), an essential substrate for *de novo* purine synthesis (Horecker et al., 1951) The HBP generates products for extra cellular matrix production and also acts as a fuel-sensing pathway. While it represents a small percentage of total glucose consumption by

the COC, the requirement for hyaluronic acid, which is formed as an end product of the HBP, during cumulus expansion ensures it holds a central role in oocyte maturation (Sutton-McDowall et al., 2010). The role of the Polyol pathway, (conversion of glucose to sorbitol and fructose), has received little attention in the COC.

Amino acid metabolism

Amino acids such as glutamine can also act as an energy source in the COC. Glutamine is metabolised to α -ketoglutarate which can then enter the TCA cycle and undergo subsequent oxidation to form ATP (Figure 1.9; Rose-Hellekant et al., 1998). Amino acids are also required for other metabolic, non-ATP producing functions such as maintaining glutathione levels (GSH). Glutathione is a potent reducing agent and ROS scavenger, the levels of which in the oocyte are closely correlated to developmental competence, due to its role in sperm decondensation and male pronucleus formation (de Matos and Furnus, 2000). It is a thiol-tripeptide constructed of cysteine, proline and glutamine therefore these precursors are necessary for adequate glutathione levels (de Matos and Furnus, 2000).

Lipid metabolism

Lipid content of oocytes varies widely between species, however triglycerides consistently account for the majority of lipid reserves (Sturmey et al., 2009). The role of this lipid in ATP provision via β -oxidation in COCs has been definitively demonstrated in many species including the pig, cow and mouse (Dunning et al., 2010; Ferguson and Leese, 2006; Sturmey and Leese, 2003). Even in species such as the mouse with relatively little lipid (oocytes appear clear, unlike large animal oocytes in which lipid droplets confer an opaque appearance) β -oxidation is essential to developmental competence (Dunning et al., 2014b).

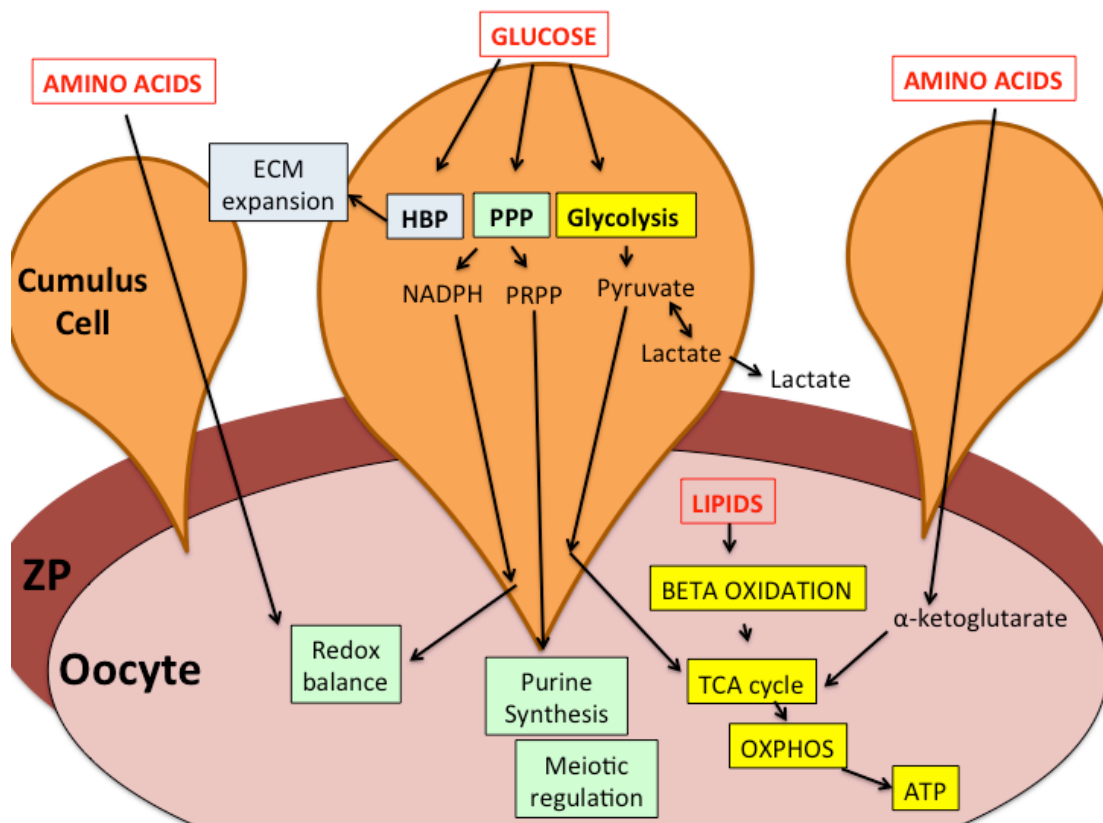


Figure 1.9: Schematic diagram depicting metabolism in the cumulus oocyte complex. (Adapted from Sutton-McDowall et al., 2010). Glucose is metabolised via three main pathways; glycolysis, pentose phosphate pathway (PPP) and the hexosamine biosynthesis pathway (HBP). Glycolysis yields pyruvate, which is transferred to the oocyte for ATP production via the tricarboxylic cycle (TCA) and oxidative phosphorylation (OXPHOS). The PPP yields NADPH (involved in redox balance) and phosphoribosyl pyrophosphate (PRPP; involved in nucleotide synthesis). The hexosamine biosynthesis pathway (HBP) produces hyaluronic acid, which contributes to extra cellular matrix (ECM) expansion. Intracellular lipids are metabolised via β oxidation and feed into the TCA cycle and amino acids such as glutamine are both oxidised after conversion α -ketoglutarate via the TCA cycle and also used for redox balance via incorporation into glutathione.

While this general understanding of energy metabolism is true for the species studied to date, the relative contribution of each pathway to ATP production and importance of each pathway to developmental competence is species-specific. Furthermore, as highlighted previously, the metabolism of the COC will adapt to its environment, which if suboptimal can disrupt the delicate balance between pathways, potentially creating a state of oxidative damage, cell dysfunction and decrease in developmental competence.

For the equine COC, the above pathways and their relative contributions to overall energy metabolism remain uncharacterised.

1.6 Conclusions

In species such as the horse, in which *IVP* is rapidly gaining commercial interest, there is an urgent need to understand the basic metabolic energy requirements of the COC, especially given the clear links between a suboptimal periconceptual environment and negative health outcomes (Harrison and Langley-Evans, 2009, Barker et al., 2002). Not only does this understanding add to our knowledge of species-specific physiology, but it also permits identification of aberrations, which subsequently creates the potential to mitigate negative downstream effects. Ultimately, this understanding can underpin the development of optimal species-specific culture media.

Whilst acceptable blastocyst rates (41%) and pregnancy rates after transfer (66%) can be achieved by some in the horse, (Hinrichs et al., 2014; Jacobson et al., 2010) the more subtle effects of potentially inappropriate culture conditions remain to be seen. Crucially, while there is a single study reporting no difference in placental and foal morphometry attributable to *IVP* (Valenzuela et al., 2017), there is no information on the long-term outcomes of the foals produced by *IVP*. In studies that have compared *in vivo* derived and *in vitro* derived embryos, consistent differences have been found such as differing morphology, delayed kinetics, decreased cell number, lack of trophectoderm differentiation, increased DNA fragmentation, increased prevalence of chromosomal abnormalities and differential gene expression (Choi et al., 2009; Hendriks et al., 2015; Pomar et al., 2005; Rambags et al., 2005; Smits et al., 2011; Tremoleda et al., 2003). While these differences could stem from inadequacies at any point in the *IVP* system, a step-wise optimization beginning with *IVM* is prudent, for which metabolic studies can form the starting blocks.

1.7 Aims and objectives of thesis

Crucial knowledge gaps identified in performing this literature review are the lack of any information on the basic metabolic energy requirements of the equine COC during IVM and the fact that the suitability of the current clinical IVM protocol has not been ascertained with many different protocols exist among laboratories. Further knowledge gaps pertain to the origin of measured differences between *in vivo* derived and IVP blastocysts. As such, the general aim of this thesis is to address these knowledge gaps by characterising carbohydrate metabolism in equine COCs during IVM and subsequently use this as a platform from which to evaluate specific medium components and environmental factors. As carbohydrates are the major contributor to energy metabolism in all species studied to date, the investigation begins by describing glucose, pyruvate and lactate (GPL) metabolism, oxidative metabolism and mitochondrial efficiency under the current clinical conditions (CHAPTER 3). The following questions will then be addressed:

- Are metabolic indices associated with markers of meiotic and developmental competence, such as nuclear maturation, cleavage, blastocyst development and embryo morphokinetics? (CHAPTER 3)
- Does varying oxygen tension or pyruvate concentration during IVM affect GPL and oxidative metabolism of COCs? (CHAPTER 4)
- Does glucose concentration during IVM, a component that significantly varies in concentration between laboratories, affect GPL and oxidative metabolism of COCs, oocyte developmental competence or morphokinetics of early embryo development? (CHAPTER 5)
- Additionally, does glucose concentration during IVM affect relative expression of genes related to metabolic enzyme activity and redox status in single oocytes and cumulus and developmental marker genes in blastocysts? (CHAPTERS 5 and 6)

Chapter 2: Materials, Methods and Method Development

Five key methodologies were used to meet the aims and objectives of this thesis; 1) *In vitro* embryo production, 2) DNA quantification assays, 3) Enzyme-linked metabolic assays, 4) Oxygen consumption rate (OCR) measurements using the Seahorse XFp analyser and 5) Gene expression analyses.

As highlighted in Chapter 1, protocols used by different laboratories can vary widely. This is especially relevant when considering *in vitro* embryo production and characterisation of metabolism. Where applicable, sections are preceded by a brief summary of the evidence-based rationale that underpinned selection of the protocols used.

2.1 *In vitro* embryo production

As a prerequisite to the reliable interpretation of oocyte and cumulus-oocyte complex (COC) metabolic data generated in these studies, it was essential to use an efficient *in vitro* embryo production (IVP) system from which developmentally competent oocytes, capable of producing viable blastocysts, pregnancies and foals could be derived. The steps involved in the IVP process will be discussed in sequence (oocyte collection and holding, *in vitro* maturation, denuding of cumulus cells and classification, intracytoplasmic sperm injection [ICSI], and embryo culture).

All procedures were conducted in agreement with the principles of the University of Liverpool Veterinary Ethics Committee and with the approval of the Royal College of Veterinary Surgeons.

2.1.1.Oocyte recovery

Oocytes for all studies were obtained from abattoir-derived ovaries. As discussed in Section 1.4.3, several variables related to ovarian transport and method of oocyte collection have the potential to impact developmental competence. These are briefly summarised below.

Evidence for consideration

Immature oocytes can be recovered from *post-mortem* ovaries by using either follicular scraping or needle aspiration techniques (Section 1.4.2). Needle aspiration, as performed in cattle IVP, is more rapid and less labour intensive compared with follicle scraping (Galli et al., 2007). However, scraping individual follicles using a bone curette (Hinrichs and DiGiorgio, 1991) yields increased recovery rates (up to 85% *c.f.* 40-60% when using the needle technique; Dell'Aquila et al., 2001). Additionally, due to the close attachment of the cumulus to the follicle wall (Hawley et al., 1995), oocytes collected by follicular scraping are recovered with a greater proportion of their associated granulosa cells. This method therefore permits a more accurate classification of the associated cumulus cell morphology when compared to aspirated COCs, which generally present with only the corona radiata intact (Dell'Aquila et al., 2001).

The evidence for an optimal ovarian transport protocol was inconclusive (Section 1.4.3). To summarise, the consensus opinion was that ovarian transport of up to 5-9 hours *post-mortem* at ambient temperature (slow cooling curve), allowed for the greatest blastocyst rates (Hinrichs et al., 2012; Ribeiro et al., 2008).

Implemented protocol

Ovaries were harvested within 15 minutes of slaughter from mares of unrecorded age and breed. Animals were slaughtered during the natural breeding season (April-October) at a UK abattoir in compliance with EU legislations EC 852/2004, 853/2004 and 854/2004, for purposes unrelated to the study. On collection, ovaries were transferred to a polythene bag within a Styrofoam container and the container was

maintained at 18-20°C (ambient temperature). At the end of the collection period (~2 hours), the container was transported to the laboratory within four hours, again at 18-20°C. On arrival at the laboratory, all visible ovarian follicles were individually incised and the granulosa layer scraped using a bone curette. Cells collected were rinsed into a Petri dish with Flush Medium (M199 with Hanks salts [Life Technologies Ltd, Paisley, UK], 0.4% Fetal Bovine Serum [FBS; Life Technologies Ltd.], 25 µg/ml gentamicin, 8 IU/ml heparin [both Sigma-Aldrich Ltd, Gillingham, Dorset, UK]). Cumulus-oocyte complexes (COCs) were identified in the recovered tissue under a dissection microscope (60-120x magnification) and transferred to a separate dish containing Manipulation Medium (M199 with Hanks salts, 10% FBS, 25 µg/ml gentamicin). Cumulus-oocyte complexes were classified as corona only (COR), compact (Cp) or expanded (Ex) on the basis of gross cumulus morphology (Section 1.4.2). COCs were classified as Ex if any expansion of the cumulus cells was evident (Hinrichs, 2010). All manipulations were conducted at 20-24°C and all ovaries were processed within nine hours of mare death.

2.1.2 Oocyte holding

Due to procedural logistics involving transport time from abattoirs and the necessity for a 30-hour maturation period, it is desirable to be able to hold oocytes in meiotic arrest overnight. This allows maturation to be initiated in the morning and thus subsequent procedures to be scheduled during daytime working hours.

Evidence for consideration

Overnight holding of immature oocytes was first described by Choi et al. in 2006. These authors demonstrated that oocytes could be maintained in meiotic arrest in the absence of meiotic inhibitors by simply holding them at room temperature in an M199-based medium containing both Earle's and Hanks' salts (EH media). Several authors have since validated overnight holding using a variety of media and holding times (range 15-39 hours) without any effect on maturation, blastocyst or foaling rates (Choi et al., 2015; Diaw et al., 2018; Dini et al., 2016; Foss et al., 2013; Galli et al., 2014a; Hinrichs et al., 2014).

Selected protocol

Recovered COCs of the same cumulus classification were collected into groups of ten and placed in individual 1-ml glass vials (Thermoscientific Inc., Waltham, MA) in EH Medium (40% M199 with Hanks salts, 40% M199 with Earle's salts [Life technologies Ltd.], 20% foetal bovine serum (FBS) and 25 µg/ml gentamicin; Choi *et al.*, 2006). Vials were sealed, wrapped in tinfoil to protect from light and held overnight (12-18 hours) at 20-24°C.

2.1.3 *In vitro* maturation

The *in vitro* maturation period is the focus of this thesis as it is central to producing a developmentally competent oocyte for fertilisation. The ideal protocol considers the media in which the COCs will be cultured but also the environmental conditions (gaseous tensions, temperature and humidity), duration of culture and technical elements such as group size. The different protocols in current use have been discussed in depth in Section 1.4.3. Below is a brief synopsis to clarify selection of the protocol used herein.

Evidence for consideration

Media selection: The two most commonly used media for equine *IVM* are TCM-199 and DMEM/F12. Both of these media have produced viable embryos and healthy offspring after ICSI and embryo culture (Galli *et al.*, 2007; Hinrichs *et al.*, 2014; Smits *et al.*, 2010). Galli *et al.*, (2007) reported that DMEM/F12 was superior to M199-based *IVM* media when considering subsequent blastocyst development (26.4% [37/140] for DMEM/F12 vs. 12% [23/191] for M199). However, an M199-based *IVM* medium containing only 10% FBS and 5mU/ml of follicle stimulating hormone (FSH) has produced the highest reported blastocyst rates (up to 42%) in numerous reports (Hinrichs *et al.*, 2005; Jacobson *et al.*, 2010; Ribeiro *et al.*, 2008; Salgado *et al.*, 2018).

Environmental conditions: These are dictated largely by the medium used. Medium containing 25 mM bicarbonate is typically used under 5% CO₂ to maintain a pH of about 7.4. M199 with Earle's salts contains 25 mM bicarbonate. There are many formulations of DMEM/F-12 available, with different concentrations of bicarbonate, and this is not typically specified in reports. The atmosphere used for IVM is typically 5% CO₂ in air, which is hyperoxic compared to the follicular environment.

Technical considerations: Small droplets of media are used to maximise the benefit of oocyte-secreted factors and thus there is a requirement for a mineral oil overlay to allow formation of the droplets. The oil overlay also minimises environmental fluctuations, such as changes in osmolality due to evaporation and changes in temperature when the dish is handled. Droplet and group size during IVM have not been critically evaluated for the horse. The majority of laboratories reporting success in equine ICSI programmes report ~10 µl/COC, with oocytes being maintained in groups of up to 50 in 500-µl droplets.

Duration of maturation: This requires careful consideration as cumulus morphology and ovarian transport time can both affect the maturation kinetics. Oocytes with compact cumulus (Cp) are reported to have greater maturation rates after immediate processing (recovery from ovaries and placement in culture) than after a delay in processing of 5-9 hours (up to 66% MII if within 45 minutes vs. 32% MII after a delay). However, time of processing did not affect maturation rate of oocytes with expanded (Ex) cumulus (Hinrichs et al., 2005). The highest blastocyst rates in that study were achieved after a delay in processing for both cumulus classifications; Ex oocytes (27-32%; after 24-36 hours maturation culture) and Cp oocytes (35-38%; after 30-36 hours maturation culture). The maturation duration reported by different laboratories is typically 26-30 hours thought to maximise developmental competence of all oocytes (Galli et al., 2016; Hinrichs et al., 2014).

Selected protocol

Maturation dishes, pre-prepared with 10- μ l droplets of media covered with an oil overlay [OVOIL™, Vitrolife, Goteburg, Sweden) were pre-equilibrated in a 5% CO₂ incubator (21% oxygen) at 38.3 °C for 12 hours before use. Classified COCs (COR, Cp, Ex) were washed in Maturation Medium (M199 with Earle's salts, 10% FBS, 25 μ g/ml gentamicin with 5 mU/ml FSH [Sioux Biochemical Inc., Sioux centre, IA] and placed in the pre-prepared droplets of Maturation Medium. When individual study objectives required modification of this standard protocol, details are provided in the relevant chapters.

2.1.4 Denuding and classification

Following the IVM period, oocytes are denuded of cumulus cells to permit visualisation of the oolemma and perivitelline space. For all species, cumulus removal is typically performed with the aid of hyaluronidase, an enzyme which dissolves the hyaluronic acid matrix. As the cumulus cells have already detached from one another and the oocyte during cumulus expansion, this allows mechanical removal of the cells.

Selected protocol

Oocytes were denuded of cumulus by repeated pipetting in a 50- μ l droplet of Manipulation Medium containing 80 IU/ml hyaluronidase (HYASE-10X™, Vitrolife). Denuded oocytes were evaluated with the aid of a dissection microscope at 500x magnification. Oocytes with a visible polar body were classified as Metaphase II (MII), oocytes with an intact oolemma but without a polar body were classified as intact and those with an irregular oolemma were classed as degenerated (DEG). (Figure 2.1)

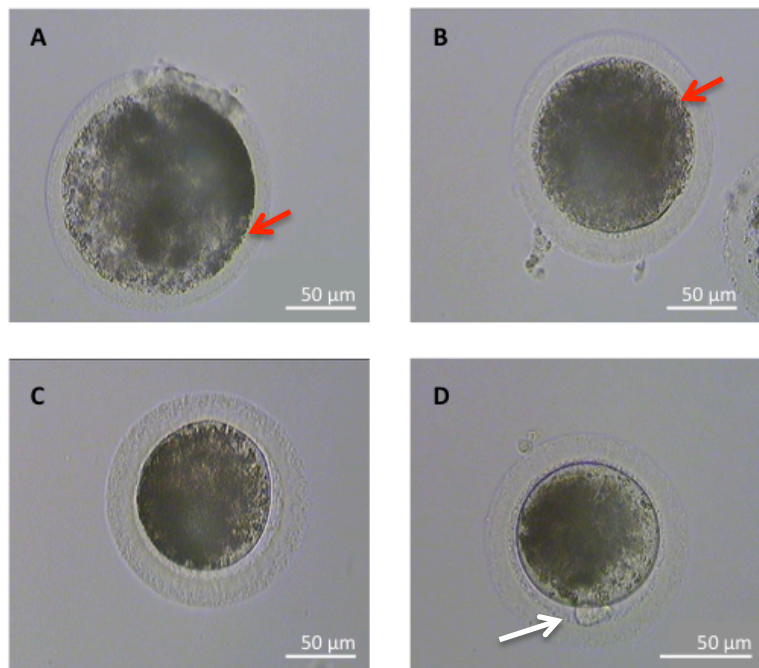


Figure 2.1: Photomicrographs of equine oocytes after cumulus cells have been denuded. Panels A and B are degenerated oocytes (DEG) highlighted by the irregularity of the oolemma (red arrow). The oocyte in panel C is classified as INTACT, due to its smooth oolemma but lack of polar body. Panel D depicts a Metaphase II oocyte (MII), with the polar body clearly visible at 6’oclock (white arrow).

2.1.5 ICSI procedure

All metaphase II oocytes are considered suitable for fertilisation by ICSI.

Evidence for consideration

ICSI involves the introduction of a single spermatozoon into the cytoplasm of an oocyte. In human laboratories this is routinely accomplished by direct penetration using a sharp micro needle (conventional ICSI). However, equine ICSI outcomes were variable and inconsistent using conventional ICSI, until reports from laboratories using the piezo drill to facilitate sperm injection in 2002 (Choi et al.,

2002; Galli et al., 2002). The piezo drill involves the use of a blunt micropipette, which delivers precise micro-electric oscillations in order to both damage the sperm plasma membrane (essential for fertilisation) and penetrate the oolemma, however mercury or other heavy liquid ballast is required to dampen the oscillations and improve precision. Use of the piezo drill greatly enhanced rates of oocyte survival and fertilisation rates in murine ICSI due to the ease of oolemma penetration, thus minimising mechanical distortion and decreasing lysis (Yoshida and Perry, 2007).

Many equine laboratories now perform piezo-assisted ICSI routinely. It has been questioned whether the piezo drill is an essential component for success in equine ICSI. One study compared laser-assisted conventional and piezo-assisted ICSI and found no significant differences between methods, however blastocyst rates were low for both (4.8% and 5.1%; Smits et al., 2012). Prior to work contained in this thesis, only a single report documented the birth of a live foal using conventional ICSI; although again, this followed low overall blastocyst rates (7.4%; Smits et al., 2010). More recently, several brief communications have reported improved blastocyst rates (> 20%) using conventional ICSI (Leisinger et al., 2016; Martino et al., 2016; Schnauffer et al., 2016). Very recently (after the completion of the studies involved in this thesis) a prospective controlled study that compared equine embryo production via conventional vs. piezo- assisted ICSI was reported (Salgado et al., 2018). These authors found no difference in blastocyst rates between the two techniques (39% and 40%, respectively) but found that blastocysts produced by conventional ICSI had lower numbers of nuclei and a higher nuclear fragmentation rate than did those produced by piezo-assisted ICSI.

It remains to be determined whether piezo-assisted ICSI offers a significant advantage over conventional ICSI in terms of embryo viability (foal production after embryo transfer). Added to this, the inclusion of the piezo drill represents a significant additional investment, introduces an additional requirement for technical expertise and presents risks for the user associated with the use of mercury (the most common ballast used). For these reasons, conventional ICSI was

used in the research involved in this thesis, as the technical expertise was already in place and therefore introduction of a learning curve was avoided.

Selected protocol

For all ICSI sessions, straws of frozen semen were from a single ejaculate from a stallion of proven fertility. A small section of one 0.5-ml straw of frozen semen was used for each session and the section was placed directly into 3 ml of warmed EMCARE™ Holding media (ICPbio Reproduction, Auckland, NZ) and incubated at room temperature (20-24°C for 15 minutes (Foss et al., 2013)). Sperm suspension from the top of the 3-ml tube was used for ICSI.

Conventional ICSI was performed using an Integra micromanipulator (Research Instruments, Falmouth, UK) by Karen Schnauffer, a senior human embryologist. A standard sharpened ICSI needle (5-µm inner diameter; Research Instruments) was used. Denuded MII oocytes were placed into individual 10-µl droplets of Manipulation Medium with addition of 0.27 mM pyruvate, and 1-2 µl of sperm suspension was placed on the left of a separate 5-µl droplet of a commercial 9% PVP solution (ICSI™; Vitrolife). Motile spermatozoa that had swum to the right of the PVP drop were immobilized by crushing of the flagellum with the ICSI needle until kinking was observed. The selected spermatozoon was aspirated into the ICSI needle, flagellum first. The oocyte was positioned with the polar body at 6 o'clock and the ICSI needle was advanced through the zona pellucida and into the oocyte cytoplasm. Puncture of the oolemma was confirmed by visualisation of cytoplasmic contents in the ICSI needle after suction and the spermatozoon was then deposited in the cytoplasm with a minimum of medium. The micromanipulator platform was maintained at 37 °C.

2.1.6 Embryo culture

Injected oocytes are cultured *in vitro*, ideally, to the blastocyst stage if they are destined for cryopreservation or transcervical embryo transfer. Culture to the blastocyst stage is also beneficial for research studies in order to determine developmental potential. Once more, many variations in culture protocols are practiced and choices are required for each of the independent factors.

Evidence for consideration

Progress in this area was slow; embryo development *in vitro* essentially stopped after 4 days (8-16 cell stage), and culture was performed either in the mare or sheep oviduct after ICSI in order to achieve blastocyst development (Choi et al., 2004; Cochran, 1998; Lazzari et al., 2010; Li et al., 2001). Early attempts at *in vitro* culture involved co-culture with a variety of somatic cells such as oviductal, Vero and cumulus cells, or trophoblast-conditioned media (Choi et al., 1994; Dell'Aquila et al., 1997; Guignot et al., 1998; Li et al., 2001). Efforts were then made to move to a semi-defined media using synthetic oviduct fluid (SOF) supplemented with BSA and amino acids (Galli et al., 2002), G1/G2 without serum (Choi et al., 2003) and CZB without serum (Choi et al., 2004), however blastocyst rates continued to be low (< 10%). DMEM/F12, a medium developed for somatic cell culture, was inadvertently discovered to support high blastocyst rates, when it was used alone as a control in a study intended to evaluate the effect of embryo co-culture with oviductal cells (18-20% blastocyst rate; Choi et al., 2004). This finding led to the widespread adoption of DMEM/F12-based media for embryo culture (Galli et al., 2007; Jacobson et al., 2010; Smits et al., 2010).

Despite the potential significance within the IVP process in the horse and defined significance in other domestic species (Wale and Gardner, 2016), the volume of culture media droplets used and/or the numbers of zygotes cultured within each droplet are rarely defined in reports of equine IVP. Droplet sizes reported in the equine literature range from 5-100 µl with 1 to 10 injected oocytes per droplet. A single controlled study evaluated the effect of DMEM/F12 droplet size (Choi et al.,

2007). These authors evaluated individual culture of injected oocytes in 2.5, 5, 10 or 50- μ l droplets and no difference was reported in blastocyst rate (21-29%). The highest blastocyst rate in that study (39%) was achieved in oocytes cultured in groups of 2-7 (average 3.2) in a fixed-size 5- μ l droplet.

Selected protocol

Injected oocytes were washed twice through pre-equilibrated Culture Medium (DMEM/F12 with 10% FBS and 25 μ g/ml gentamicin; Hinrichs et al., 2005) and were placed in individual wells (approx. 2- μ l volume) of a Primovision™ “well of the well” system dish (individual microwells under a 50- μ l overlay droplet; Vitrolife) under oil at 38.3°C in 5% CO₂, 5% O₂ and 90% N₂. Dishes with media and oil overlay had previously been pre-equilibrated for a minimum of six hours. On Day 4 after ICSI, medium in the original droplet was refreshed by adding 20 μ l of fresh Culture Medium and then removing 20 μ l. Injected oocytes were continuously monitored using the Primovision™ Time-Lapse System. Embryo developmental stage was determined on review of the time-lapse images. Cleavage was defined as occurring at the time of the first visually identifiable indentation of the oolemma. Embryos were classified as blastocysts on first appearance of apparent formation of a central fluid-filled cavity (blastulation).

2.1.7 Proof of concept

In separate studies (by the same author but unrelated to this thesis), individual aspects of the IVP process were evaluated and validated (Lewis et al., 2016; Appendix 2). Blastocyst rate, using the selected protocols listed above, was 7.2% and the first of several live foals was produced before the studies outlined in this thesis were initiated (<https://www.bbc.co.uk/news/av/science-environment-34576685/could-ivf-save-rare-british-breeds>). While a blastocyst rate of 7.2% is lower than those reported by the limited number of leading laboratories (20-40%; Foss et al., 2013; Galli et al., 2007; Hinrichs et al., 2014), it is similar to those reported by other established groups (Galli et al., 2014a; Smits et al., 2010).

Key to this thesis is that the culture protocols used to generate data have been validated in terms of their ability to produce developmentally competent oocytes and embryos.

2.2 Enzyme-Linked metabolic assays

In the course of these studies metabolic consumption and release assays (CoRe; Guerif et al., 2013) were carried out to detect depletion and/or appearance of key substrates (glucose, lactate, pyruvate) in spent media. Assays were based on the ultra-fluorometric enzyme-linked assays first described by Leese and Barton, (1984). All measurements were carried out on a FLUOstar Omega spectrophotometer (BMG Labtech; Buckinghamshire, UK) set to excite samples at 340 nm and collect emission spectra at 460 nm using V-bottom, clear 96-well plates (Star lab, UK).

Whole /VM culture dishes containing both reference droplets (droplets of medium without COCs) and empty maturation droplets, which had been stored at -80 °C following removal of COCs under investigation, were thawed immediately prior to the assay. The enzyme reaction mixture (prepared prior to the assays and stored at -20 °C for up to 3 months) was added to the 96-well assay plate first (mixture and volume detailed below for each substrate assay) and background fluorescence was measured. Several sample dilutions (diluted with molecular grade water; 18.2 Ω) were tested for each substrate assay in order to identify the optimum dilution factor that gave the lowest co-efficient of variation (CV < 10%), while conserving the initial sample volume. The diluted sample medium was then added to the enzyme reaction mixture and incubated for the period of time dictated by the substrate-specific assay being performed (see sections 2.2.1-3), after which the change in fluorescence was recorded. This change in fluorescence was based on enzymatic reduction of NADP⁺ (glucose), NAD⁺ (lactate) or enzymatic oxidation of NADH (pyruvate; see equations below). All measurements were performed in triplicate (3 wells per sample), corrected for the initial background fluorescence and calibrated against a 6-point standard curve of known standards, prepared before each individual assay.

Any standard curve giving an r^2 value of less than 0.99 was rejected, and the standard curve repeated before any samples were analysed. The mean of the empty reference droplet readings was subtracted from each sample reading and the mean of the triplicate measures (3 measures of same sample) calculated. Values were only retained if the technical triplicate CV was < 10%. Data were expressed as substrate consumed/produced in pmol/COC/hour. Later this was adapted to account for cumulus cell number (see Section 2.3).

2.2.1 Glucose Assay

Background fluorescence measurements were taken by adding 9 μl of enzyme reaction mixture to the assay plate (Table 2.1). Sample maturation medium was prepared as a 1:6 dilution with molecular grade H_2O and reference maturation medium 1:10 to ensure values obtained were within the range of the 0- to 1-mM standard curve. Then, 1 μl of diluted sample/reference medium was added to the enzyme reaction mixture and the assay plate incubated for 10 minutes at 37°C prior to reading.

Glucose concentrations were determined by the reduction of NADP^+ , which results in an increase in fluorescence as shown below:



The reaction mixture was prepared prior to the assays and stored at -20°C for up to 3 months as follows: 15 ml EPPS buffer at pH 8 (Table 2.1), 1 ml hexokinase (HK)/glucose -6- phosphate dehydrogenase (G6PDH) [final concentration 20IU/ml], 2 ml Dithiothreitol stock, 2 ml Magnesium Sulphate stock, 3 ml NADP stock and 1 ml ATP stock.

	Chemical	Weight (mg)	H_2O added (ml)	Final concentration	Manufacturer
EPPS buffer	EPPS	2520	150		Sigma
	Penicillin G	10		50 $\mu\text{g}/\text{ml}$	Sigma
	Streptomycin	10		50 $\mu\text{g}/\text{ml}$	Sigma
	Sodium hydroxide	To effect			Fisher
Stock solutions	Dithiothreitol	7.715	10	0.4 mM	Sigma
	Magnesium sulphate	91.2	10	3.07 mM	Sigma
	NADP	39.37	5	1.25 mM	Roche
	ATP	30.26	5	0.42 mM	Sigma

Table 2.1: Chemical composition of the glucose assay enzyme reaction mixture.

2.2.2 Lactate assay

Background fluorescence measurements were taken by adding 9 μl of reaction mixture to the plate (Table 2.2). Sample maturation medium was prepared as a 1:6 dilution with distilled H_2O and reference maturation medium 1:10 to ensure the values obtained were within the range of the 0 to 1-mM standard curve, then 1 μl diluted sample/reference medium was added to the enzyme reaction mixture and the plate incubated for 30 minutes at 37°C .

Lactate concentrations were determined by the reduction of NAD^+ by lactate dehydrogenase (LDH) resulting in an increase in fluorescence as shown below:



The reaction mixture was prepared prior to the assays and stored at -20°C for up to 3 months as follows: 9 ml Glycine buffer at pH 9.4-(Table 2.2), 8 ml dH_2O , 1.5 ml of NAD stock (final concentration 4.8 mM) and 0.5 ml LDH (final concentration 40 IU/ml).

	Chemical	Weight (g)	H_2O added (ml)	Manufacturer
Glycine buffer	Glycine	7.5	100	Sigma
	Hydrazine sulphate	5.2		Sigma
	EDTA	0.2		Sigma
	Sodium Hydroxide	To effect		Fisher
Stock solutions	NAD	0.4	10	Roche

Table 2.2: Chemical composition of the lactate assay enzyme reaction mixture.

2.2.3 Pyruvate assay

Background fluorescence measurements were taken by adding 18 µl of reaction mixture to the plate (Table 2.3). Sample maturation medium and reference maturation medium samples were undiluted to ensure values obtained were within the range of the 0 to 0.45-mM standard curve, then 2 µl of sample/reference medium was added to the enzyme reaction mixture and the plate incubated for 3 minutes at 37°C.

Pyruvate concentrations were determined by the oxidation of NADH using the enzyme LDH, which results in a decrease in fluorescence as shown below.



The reaction mixture was prepared prior to the assays and stored at -20 °C for up to 3 months as follows: 14 ml EPPS buffer at pH 8 (as per Table 2.1), 0.3 ml of NADH stock (final concentration 0.1 mM) and 0.4 ml LDH (final concentration 40 IU/ml).

	Chemical	Weight (mg)	H ₂ O added (ml)	Manufacturer
EPPS buffer	As for glucose reaction			
Stock solutions	NADH Di sodium salt	17.74	5	Roche

Table 2.3: Chemical composition of the pyruvate assay enzyme reaction mixture.

2.2.4 Final selected protocols

It was necessary to establish a protocol by which glucose consumption, lactate production and pyruvate production by single equine COCs could be evaluated over the entire course of *IVM* (30 hours). In addition, it was desirable to perform sequential incubations for shorter periods in order to evaluate the temporal changes in metabolism during the 30 hours of *IVM*. Evaluating temporal changes would allow insight into the pathways used by the COC for each phase of oocyte maturation, such as resumption of meiosis and cumulus expansion.

The majority of equine systems use group culture for *IVM*, however this approach was inappropriate for meeting the study aims of assessing substrate depletion/accumulation of single COCs. A priority was therefore to evaluate the impact of single as opposed to group culture of COCs on the meiotic competence of the oocytes. In a preliminary study, rates of progression to metaphase II were compared between COCs incubated in conventional group culture ($n = 80$, 10 COCs/100- μ l droplet) and in individual culture ($n = 36$, 1 COC/10- μ l droplet). Metaphase II (MII) rates did not differ between the two groups (53% [42/80] for group culture and 47% [17/36] for individual culture; $p > 0.05$), thus validating the use of individual culture.

Protocol 1- Continuous incubation

COCs collected by follicular scraping were incubated continuously for 30 hours individually in 10- μ l media droplets. Twelve 9- μ l droplets of media were arranged on each culture dish (35-mm Falcon™; Hunter Scientific, Essex, UK) with a 3.5-ml oil overlay (OVOIL™; Vitrolife) as per the schematic below (Figure 2.2). COCs were washed in maturation medium. In each dish, a single COC in 1- μ l of medium was introduced into each of nine droplets. The remaining three droplets per plate had 1- μ l blank maturation medium introduced only. These droplets acted as internal reference control droplets. At the end of the incubation, COCs were removed in a minimum of medium and dishes stored at -80 °C until spent media analyses were carried out.

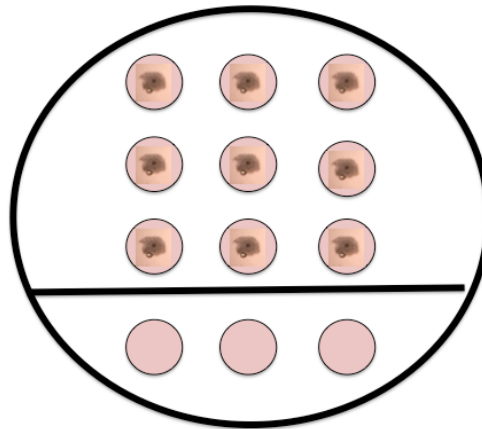


Figure 2.2: Schematic depicting the standard layout of the IVM culture dish. Single COCs occupied nine of the droplets and three droplets remained empty to act as internal reference droplets. All droplets had a final volume of 10 μ l and the dish had a 3.5-ml oil overlay.

Protocol 2- Discontinuous sequential incubation

COCs collected by follicular scraping were incubated for three consecutive 10-hour periods. Dish set up was identical to Protocol 1, however after each 10-hour incubation period, COCs were moved, in 1 μ l of medium, to the same droplet location in the next dish (Figure 2.3). At the end of the incubation, COCs were removed in a minimum of medium and dishes stored at -80 °C until spent media analyses were carried out.

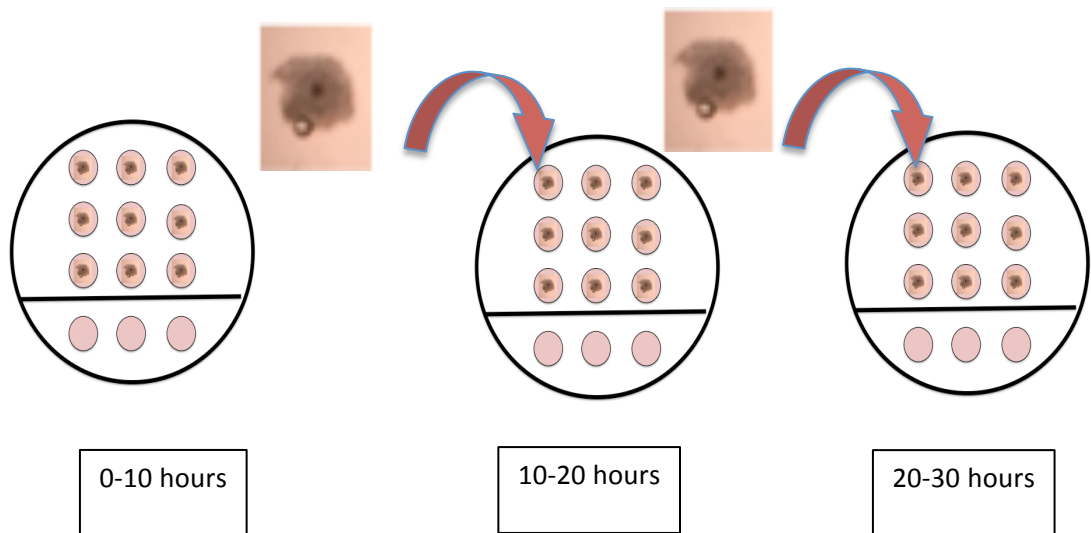


Figure 2.3: Schematic depicting the standard layout of IVM culture dishes for sequential incubation. Single COCs occupied nine of the droplets and were moved every 10 hours to the same location in the next dish. Three droplets remained empty on each dish to act as internal reference droplets. All droplets had a final volume of 10 μ l and the dishes had a 3.5-ml oil overlay.

2.3 DNA quantification of cumulus and granulosa cells

A pronounced variation in amount of cumulus cells was noted between COCs, which could greatly affect the perceived metabolic rate of the COCs. Different degrees of expansion rendered visual appraisal or morphometry inaccurate, thus a quantitative DNA assay was used as a proxy for the number of cumulus cells per COC. This permitted the normalisation of substrate and oxygen consumption rates between COCs.

On completion of incubations, COCs were individually denuded, and recovered cumulus cells from each COC were placed directly into a single well of a 96-well plate (VWR International, Leics, UK) and stored at -20 °C pending analysis. DNA was subsequently extracted from thawed cells using the method described by (M L Sutton et al., 2003). Briefly, this entailed the addition of 50 µl/well of lysis buffer (50 mM Tris- HCL, 1mM EDTA, 500µg/ml Proteinase K; all Sigma). Plates were then incubated for 180 minutes at 50 °C, followed by a further 10 minutes at 80 °C to deactivate Proteinase K. To eliminate background fluorescence associated with the presence of RNA, 50 µl of DNase free RNase (20 µl/ml; Roche Diagnostics Ltd, West Sussex, UK) was added to each well and plates were further incubated for 30 minutes at 40°C. Excess supernatant was removed by evaporation at room temperature overnight.

The following day, 100 µl of Tris- EDTA buffer (Quant- IT™, Picogreene®, Life Technologies) was added to each well, and a standard curve (0-200 ng DNA/well) was prepared on the same plate with ds-lambda DNA standard diluted in Tris- EDTA buffer (Quant- IT™, Picogreene®, Life Technologies). An initial background fluorescence reading was recorded (excitation 480 nm and emission 520 nm; Glomax® Multi detection system, Promega, Suffolk, UK) before 100 µl of Picogreene solution (200-fold dilution, Quant- IT™, Picogreene®, Life Technologies) was added to all wells. The plate was incubated while protected from light for 3 minutes at room temperature before final fluorescence was recorded. The background reading was subtracted and the final fluorescence plotted against the standard curve to

ascertain the quantity of DNA per well in nanograms (ng). All standard curves had an r^2 of ≥ 0.99 .

2.4 Oxygen consumption rate measurement

Oxygen consumption rate (OCR) and contribution of oxidative phosphorylation (OXPHOS) to ATP production have not previously been evaluated for equine oocytes, COCs or embryos. Oxygen consumption was first described in rabbit oocytes by Fridhandler et al., (1957) who used a Cartesian diver technique. Since then, several methods have been reported, all with various shortcomings. Spectrophotometry was described in 1986 by Magnusson et al., however this method lacked sensitivity. Pyrene has also been used as a fluorescence marker (Houghton et al., 1996; Sturmey and Leese, 2003; Thompson et al., 1996), however this method is a closed system and relies on prolonged measurements on groups of oocytes. More recently, Lopes et al., (2005) used nanorespirometry for individual bovine embryos. This technique, which while very sensitive, requires extensive handling, and analysis in a separate environment that differs from that in which they are cultured.

The Seahorse XFp analyser (Agilent technologies LDA UK Limited, Cheshire, UK) allows the precise and non-invasive measurement of OCR by measuring changes in oxygen tension (mmHg) over time (Figure 2.4B). It does this by creating a transient microchamber of approximately 2 μ l around cells placed in a monolayer at the bottom of each individual well (Figure 2.4A). The change in oxygen tension over time is then converted to OCR as pmol/well/min using a proprietary algorithm (Gerencser et al., 2009). While its application has not yet been reported for oocytes or COCs, its non-invasive and sensitive nature renders it ideal for the objectives of this research. Additionally, the Seahorse XFp analyser has the ability to automatically introduce up to four respiratory inhibitors sequentially during undisturbed culture.

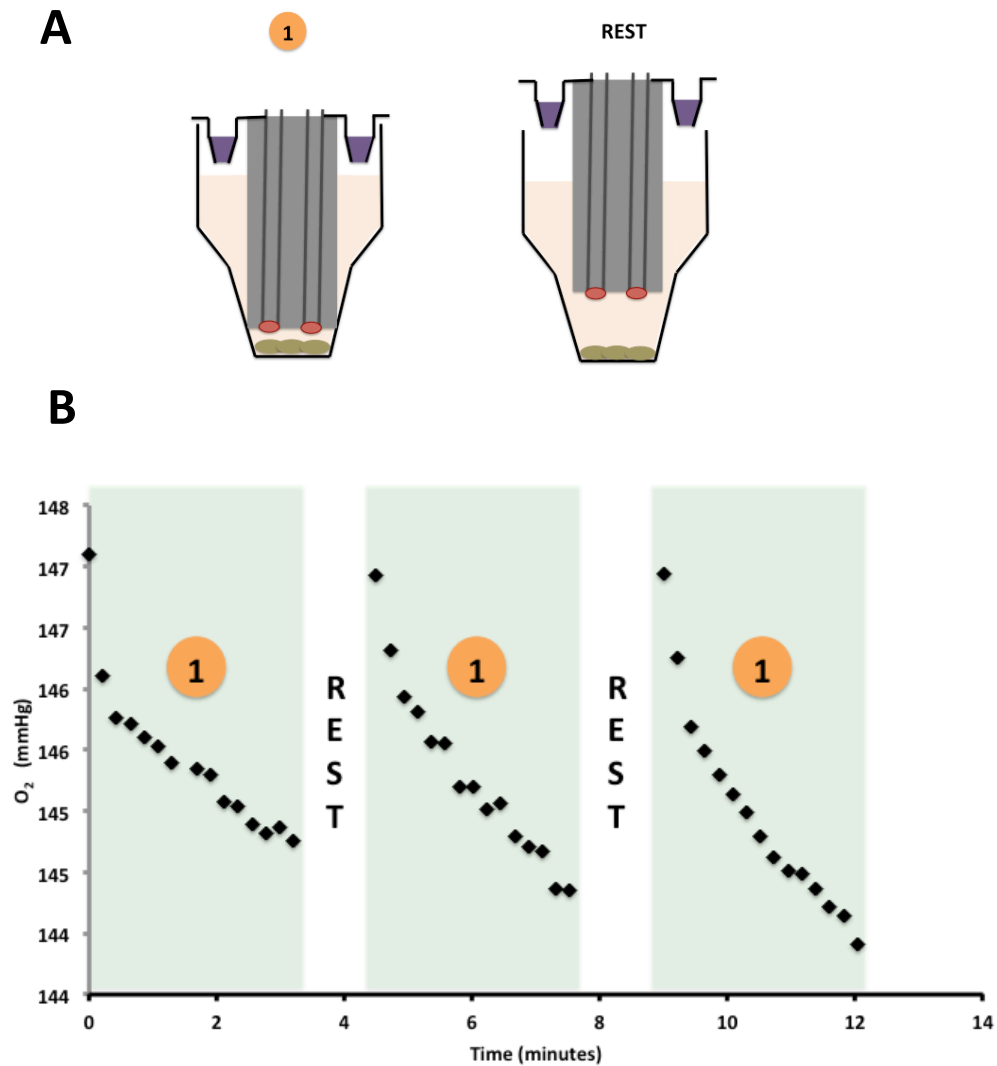


Figure 2.4: Schematic diagrams depicting the method used by the Seahorse XFp analyser to evaluate oxygen consumption rate. Panel A shows an individual Seahorse well during three-minute measurement phase (sensor cartridge probe down; 1) and during one-minute rest phase (sensor cartridge probe up; REST). Wells are filled with medium and cells are placed at the bottom of the well (brown). Oxygen tension (mmHg) is read by the fluorophore probe sensors (red) located at the bottom of the probe. Injection ports are located in the sensor cartridge of the plate, which fits as a lid during experiments and these can be filled with respiratory inhibitors if required (purple). Panel B depicts oxygen tension decreasing over time during three-minute measurement phase (1) and the one-minute rest phase allowing oxygen tension to return to atmospheric 152 ± 10 mmHg. A technique-specific algorithm then converts the change in oxygen tension over time into oxygen consumption rate in pmol/well/minute.

2.4.1 Approach to determining mitochondrial function

As described in Chapter 1 (Section 1.5.2), evaluating separate aspects of mitochondrial function gives greater insight into mitochondrial efficiency than does basal OCR alone. To do this, OCR coupled to ATP production, that associated with non-mitochondrial processes, proton leakage, and maximal OCR are calculated by the sequential use of respiratory inhibitor compounds (Figure 2.5 and 2.6). Oligomycin, an inhibitor of ATP synthase, is added first and decreases OCR to that not linked to ATP synthesis by oxidative phosphorylation. FCCP is a proton ionophore which acts to uncouple the flow of protons across the mitochondrial membrane from the normally restrictive rate of passage through Complex V (ATP synthase) of the electron transport chain. In essence, this indicates the maximal rate of OCR; the difference between this value and resting OCR is the “spare capacity” – the bioenergetic ‘top speed’ that the system can operate at. Antimycin and Rotenone are added in combination at the end. They inhibit the quinone reduction (Q_i) site in Complex III and Complex I of the electron transport chain, respectively, thereby blocking all OXPHOS from taking place in the mitochondria. The proportion of OCR that is insensitive to the addition of antimycin and rotenone is that consumed by non-mitochondrial processes.

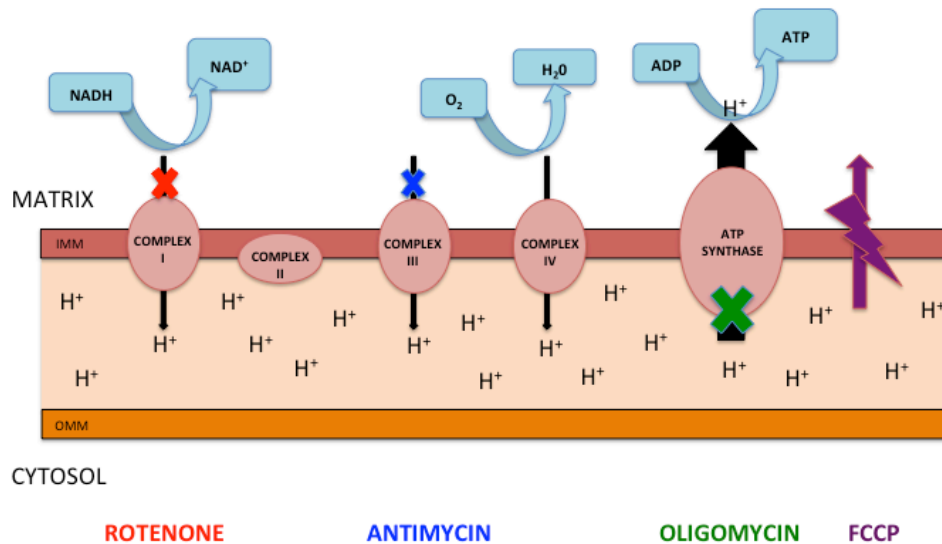


Figure 2.5: Schematic diagram of electron transport chain showing site of action of each respiratory inhibitor (Rotenone inhibits Complex 1 [RED]; Antimycin inhibits Complex 3 [BLUE]; Oligomycin blocks ATP synthase [GREEN] and FCCP damages the inner mitochondrial membrane [IMM; PURPLE]). Abbreviations are as follows; ATP = Adenosine triphosphate, ADP = adenosine diphosphate, NAD = nicotinamide adenine dinucleotide, NADH = reduced NAD, OMM =outer mitochondrial membrane.

The following equations are used to calculate the individual measures of mitochondrial function and highlighted in the same colour scheme in the schematic below (Figure 2.6).

(1) Basal OCR – OCR after Oligomycin = **OCR coupled to ATP production**

(2) OCR after FCCP = **Maximal respiration**

(3) Maximal respiration- basal OCR = **Spare respiratory capacity**

(4) OCR after Antimycin/Rotenone = **Non mitochondrial respiration**

(5) OCR after Oligomycin - non mitochondrial OCR = **Proton leak**

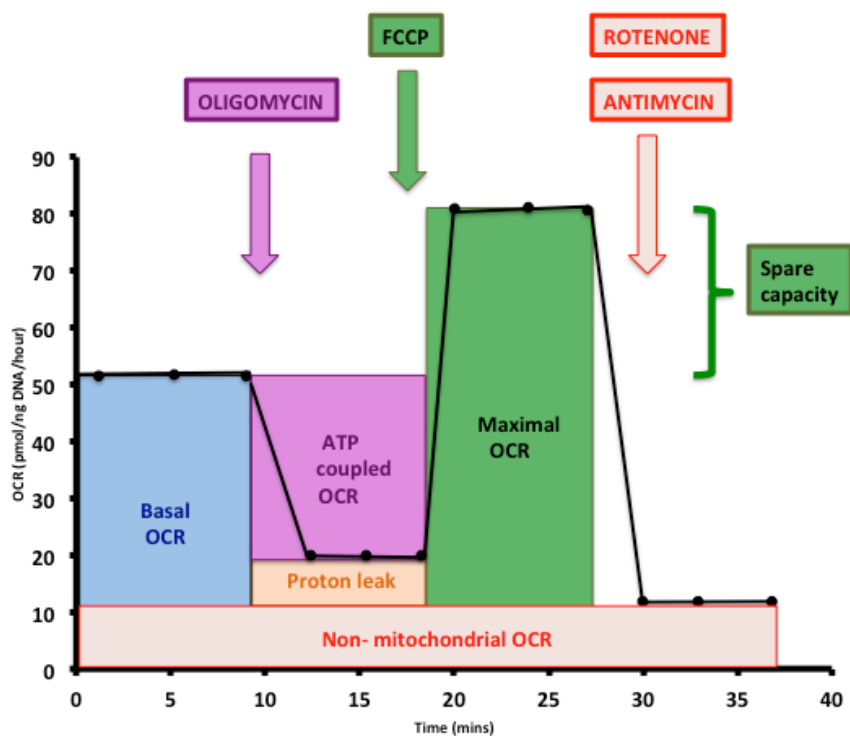


Figure 2.6: Schematic diagram to clarify how each measure of mitochondrial function is calculated using respiratory inhibitors. (Adapted from agilent.com)

2.4.2 Optimisation required for OCR measurement in COCs

In order to establish whether the Seahorse XFp analyser could be used to measure OCR in COCs, the following variables were evaluated and optimised; Group size, inhibitor concentration and incubation media. Preliminary investigations were carried out by myself and Bethany Muller using bovine COCs, due to the limited availability of equine tissue

COC group size

OCR was measured in wells containing COCs, either singly or in groups of two or groups of three. Single COCs did not consistently yield reliable values for OCR, while groups of three COCs resulted in the most consistent rates with the least variation. Oocytes with a corona radiata layer only were evaluated in groups of three or six; groups of six resulted in the most consistent OCR.

Inhibitor concentration selection

Respiratory inhibitors must be diluted in ethanol to ensure stability. As ethanol can be toxic to cells, it was first necessary to ascertain that the final concentration of ethanol in each well did not damage cell function. In preliminary studies, it was found that concentrations of analytical-grade ethanol up to 1% v/v in individual wells had no measurable impact on OCR in bovine COCs. As maximum ethanol concentrations during any study in the current thesis were 0.064% v/v, this established the lack of effect of the concentration of ethanol used.

A series of titration experiments, using bovine COCs, were performed to determine the optimum concentrations of each of the metabolic inhibitors (optimal defined as that which elicited maximal response while allowing the subsequent inhibitors to have an effect). As a result, the following concentrations were selected: Oligomycin 1 μ M; FCCP 5 μ M; Antimycin/Rotenone 2.5 μ M (All Sigma). When these selected concentrations were added to equine COCs, the response was within the observed range for bovine COCs.

Incubation medium

Conventional assays using the Seahorse XFp analyser use unbuffered medium to allow the simultaneous measurement of extracellular acidification. However, since this was not an endpoint under investigation in these studies, an *IVM* medium known to support developmental competence was chosen (bicarbonate-buffered M199 with Earle's salts and 10% FBS). The use of this bicarbonate-buffered medium resulted in the pH rising to 7.9 during a 40-minute analysis period, therefore in complimentary experiments on bovine COCs, phosphate + HEPES-buffered (stable pH) and bicarbonate buffered medium were compared. Bovine COC OCR did not vary between the two media (data not presented). Additionally, bicarbonate buffered M199 medium allowed OCR to be measured *in situ* during IVM without excessive handling of the COCs. When tested the increase in pH during the analysis period did not appear to impact OCR over time or MII (40%) rate in equine COCs (n = 12; Figure 2.7).

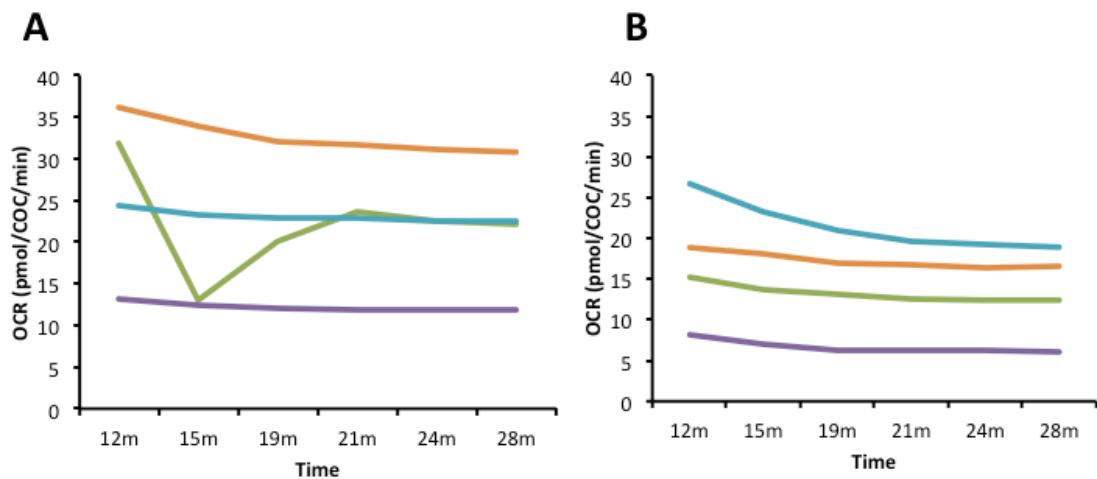


Figure 2.7: Oxygen consumption rate (OCR) measurements over time of equine COCs incubated in M199 with Earle's salts. Each line represents the average OCR per COC in each well. Measurements were taken at two times during maturation; four hours (A) and 28 hours (B) after initiating in vitro maturation. Each colour represents the same well at the two time points. Readings were begun after a 12-min equilibration phase.

2.4.3 Final protocol selected

Oocyte collection and holding was performed as described in Sections 2.1.1 and 2.1.2. On the morning maturation culture was due to begin, each well of an 8-well Seahorse assay plate was filled with 170 μ l of pre-equilibrated Maturation media (M199 with Earle's salts, 10% FBS, 25 μ g/ml gentamicin with 5 mU/ml FSH) unless otherwise stated in individual experiments. External wells were filled with 400 μ l of distilled water to provide humidity and the plate was placed in a 5% CO₂ incubator at 38 °C for a minimum of two hours. Three COCs in 10 μ l of Maturation medium were loaded into each of six individual wells, and 10 μ l Maturation medium only was placed in the remaining two wells (final volume of each well 180 μ l). The loaded plate was then placed back in the incubator with the lid on. Seahorse sensor cartridges were hydrated for a minimum of 12 hours before use at 37 °C by placing 200 μ l of calibrant solution in each well and 400 μ l of distilled water in each external well.

Basal OCR measurements

To measure OCR of COCs the sensor cartridge was placed in the Seahorse XFp analyser, then the culture plate was removed from the incubator and also placed in the Seahorse XFp analyser [preheated to 37°C]. After an initial 12-minute equilibration phase, measurements were taken over three minutes (15 readings per measurements with a 90 second rest period between measurements (Figure 2.4). Basal reading experiments were performed in less than 30 minutes, after which the culture plate was placed back in the incubator.

Mitochondrial function testing

All inhibitors were prepared with the same medium contained in the well into which they were to be injected. The sensor cartridge compound injection ports for all eight wells were first loaded with the following; Port A: 20- μ l 10 μ M Oligomycin; Port B: 22- μ l 50 μ M FCCP; Port C: 25 - μ l 25 μ M Antimycin/Rotenone. The volumes injected resulted in a dilution factor of 10 in the well medium. Therefore, final concentrations of each inhibitor in the well were: 1 μ M Oligomycin; 5 μ M FCCP; 2.5 μ M Antimycin/Rotenone.

The sensor cartridge and culture plate were then placed in the Seahorse XFp Analyser and after an initial 12-minute equilibration phase, three basal measurements were taken (as above) before each inhibitor was injected sequentially. Three further measurements were recorded after each injection. The total analysis time was less than 90 minutes, after which the COCs were removed for DNA analysis and the culture plate discarded.

2.5 Gene expression analysis

Throughout investigations for this thesis, the emphasis was on tracking development and metabolism of single COCs. As such, a method for analyzing transcript abundance in single oocytes was required. Methods that have been used for RNA amplification from small samples of oocytes and/or single oocytes/blastocysts include linear amplification, which is an *in vitro* transcription based protocol (Shaw et al., 2013; Smits et al., 2009) or exponential PCR based protocols, with linear amplification being less prone to amplification artifacts (Patrizio et al., 2007). PolyA PCR is a transcription-based method, in which an Oligo-DT primer is annealed to the mRNA polyA tail and reverse transcriptase is added to generate the cDNA. A polyA tail is then added and the PolyA-tailed cDNA is amplified through serial linear amplifications. The initial Oligo-DT has 24 'A' nucleotides therefore would detect and anneal to most if not all of the stored and polyadenylated oocyte mRNA (range 18-250 'A' nucleotides).

PolyA PCR preserves the relative abundance of transcripts within each sample and avoids biased amplification due to using a very limited reverse transcription step, thereby ensuring all products are between 300-600 bp in length and at the 3' UTR. This method also optimizes the amount of sample available as it avoids the requirement for RNA purification step (Bloor et al., 2002; Brady and Iscove, 1993; Núnêz et al., 2000). However, limitations include not being able to access any splice variants in the gene, which would be at the 5' UTR and also primers for both genes of interest and reference genes need to be 3' end directed. PolyA PCR has thus far not been reported for equine samples and therefore required development and validation for single equine oocytes, cumulus/granulosa cells and single blastocysts. Additionally, stable 3' end directed gene primers required identification and validation for use in the cDNA samples. Despite the equine genome being sequenced, very few genes are annotated the entire way to the 3'end. As such primer design presented a challenge.

Furthermore, given the existing challenges and the heterogeneity of the oocyte population, identifying stable reference genes to which data could be normalized against was essential. As there has been a lack of robust reference gene validation in the equine literature for oocytes or cumulus cells, this was a priority. For blastocysts, there have been two studies carried out to identify stable reference genes, one in a range of early developmental stages (morula to expanded blastocyst; Paris et al., 2011) and one in *in vivo* vs. *in vitro* produced blastocysts (Smits et al., 2009). However, as each culture system varies and the PolyA PCR amplification technique was unique, it was prudent to also validate blastocyst reference genes in our own experimental system.

2.5.1 PolyA PCR protocol

Lysis and cDNA amplification

The PolyA PCR technique was used as previously described by Bloor et al., (2002) and Brady and Iscove, (1993) . Briefly, the sample oocyte, cumulus cells or blastocyst were first placed in individual tubes containing 10- μ l lysis buffer (2- μ l RT enzyme buffer [ThermoFisher Scientific], 40 μ g/ml BSA, 0.5% NP40, 9.7 pmol/l dNTPs, 23 pmol/l Not1 oligo dT₂₄ [all Roche] and 0.8 units/ μ l RNAsin ribonuclease inhibitor [Promega]). The tubes were then heated to 65°C for 1 minute followed by cooling at 25°C for 3 minutes then placed on ice, which allowed unfolding of the mRNA and subsequent annealing of the Not 1 oligo dT₂₄ to the polyA tail. Reverse transcriptase (25u; M-MLV; Thermofisher Scientific) was then added to each tube and the tubes placed in a thermal cycler first at 37°C for 15 minutes followed by 65 °C for 10 minutes, then again cooled on ice. This restricted cycle length ensured extension of the first strand to between 300-600 base pairs.

Tailing buffer (10 μ l; 3.5 μ l TdT enzyme buffer [ThermoFisher Scientific], 0.16mmol/l dATP [Roche] and 0.45 units/ μ l rTdT [Invitrogen]) was added to each tube of newly synthesized 1st strand cDNA, heated to 37°C for 15 minutes followed by 65 °C for 10 minutes, then again cooled on ice. This step added a polyA tail to the 1st strand cDNA, which then allowed subsequent amplification.

Primary amplification took place by the addition of 5- μ l tailed first strand synthesis cDNA product to 10- μ l primary amplification mastermix (2.6 μ l 10x PCR buffer [Roche], 2mmol/l dNTPs, 0.04mg/ml BSA, 0.2% Triton X-100, 6.3mmol/l $MgCl_2$ [both Sigma Aldrich], 8.5 μ mol/l Not1 oligo dT₂₄ and 0.16IU/ μ l Taq DNA Polymerase [Roche]). The following cycle profile was then used; 29 cycles of 1 minute at 94 °C, 2 minutes at 42 °C and 6 minutes at 72 °C followed by a further 29 cycles of 1 minute at 94 °C, 2 minutes at 42 °C and 2 minutes at 72 °C.

Secondary amplification was then performed using 1- μ l primary amplified cDNA product added to 49- μ l secondary amplification mastermix (5 μ l PCR buffer, 0.2 mmol/l dNTPs, 2 μ mol/l Not1 oligo dT₂₄ and 0.025 IU/ μ l Taq DNA Polymerase. The following cycle profile was then used; initial denaturation at 94 °C for 1 minute followed by 70 cycles of 30 seconds at 94 °C, 30 seconds at 54 °C and 30 seconds at 72 °C. Holding was carried out at 4 °C and then samples were stored at -20 °C until gene specific PCR was carried out.

Picogreene cDNA quantification

Secondary amplification samples were thawed and 5- μ l diluted 1:200 using Tris-EDTA buffer (Quant-IT™, Picogreene®, Life Technologies). A standard curve (0-1000 ng/ml) was also prepared with ds-lambda DNA standard and Tris-EDTA buffer (Quant-IT™, Picogreene®, Life Technologies). Samples and standards (100 μ l) were added to the plate in triplicate and an initial background fluorescence reading was taken (excitation 480nm and emission 520nm; Glomax® Multi detection system, Promega). After addition of 100- μ l Picogreene solution to each well (200 fold dilution, Quant-IT™, Picogreene®, Life Technologies), the plate was incubated protected from light for 3 minutes at room temperature. Fluorescence was then measured again. The background reading was subtracted and the final fluorescence plotted against the standard curve to ascertain cDNA concentration. Standard curves were only used if $r^2 > 0.99$. Each secondary amplification product was then diluted to 1ng/ μ l concentration using PCR grade water and stored again at -20 °C.

2.5.2 Reference gene selection

A total of six reference genes were selected for stability testing based on either their validated use in other equine tissues or as validated on a wide range of tissues in other species (de Jonge et al., 2007; Paris et al., 2011; Smits et al., 2009). SYBR® primers were designed within the 300bp immediately upstream from the polyA tail at the 3' UTR (Primerdesign LTD; Table 2.4). All primers were bio-informatically validated and QC tested by Primerdesign LTD.

Name	Accession number	Sense primer	Antisense primer
RPL32	XM_001492042.5	CGGAAGTTCCTGGTCCACAA	GTGAGCAATCTCAGCACAGTAC
UBC	NM_0010818621	CGACCAGCAGAGGCTCATC	TGGACATTACAAAGACAAGACT GAA
SRP14	XM_001503583.4	AGCTAGGTTTCTGGTTCTCCT AC	AACTGGCTTCTTACAACCTCT
HPRT 1	XM_014729196.1	ACATATCAGTGACAGCATCT AAGAG	TGAACAAGTGGGAAAATACAGT TAAT
ACTB	NM_001081838.1	CGACAGGATGCAGAAGGAG AT	GTGGACAATGAGGCCAGAATG
SDHA	XM_014734954.1	ATGTGTCCTGGAGTATTCAA GTAAC	TTGATTGGATTCTACAGAAGCG

Table 2.4: Genes and SYBR® primer sequences used for reference gene stability testing.

Reference gene selection was performed using cDNA from six oocyte and six cumulus cell samples (chosen at random; one from each experimental group post IVM and two pre-IVM See Section 6.3.1). For blastocysts, cDNA from all five that were produced (See Section 5.4.1) were used. qPCR reactions were made up as follows and plated in duplicate; 5 µl cDNA (5ng), 1 µl primer, 10 µl PrecisionPlus mastermix (Primerdesign LTD) and 4 µl PCR water. Amplification was performed on a Roche Lightcycler 480 using the following protocol; initial enzyme denaturation of 2 minutes at 95 °C then 40 cycles of 10 seconds at 95°C and 60 seconds at 60°C. Post PCR melt curves were run for each gene to test specificity and the gene excluded from analysis if more than one peak existed (Figure 2.8). The two most

stable genes were then chosen (and redesigned in Taqman® probe format by Primerdesign LTD) to run alongside the target gene qPCR for further stability testing.

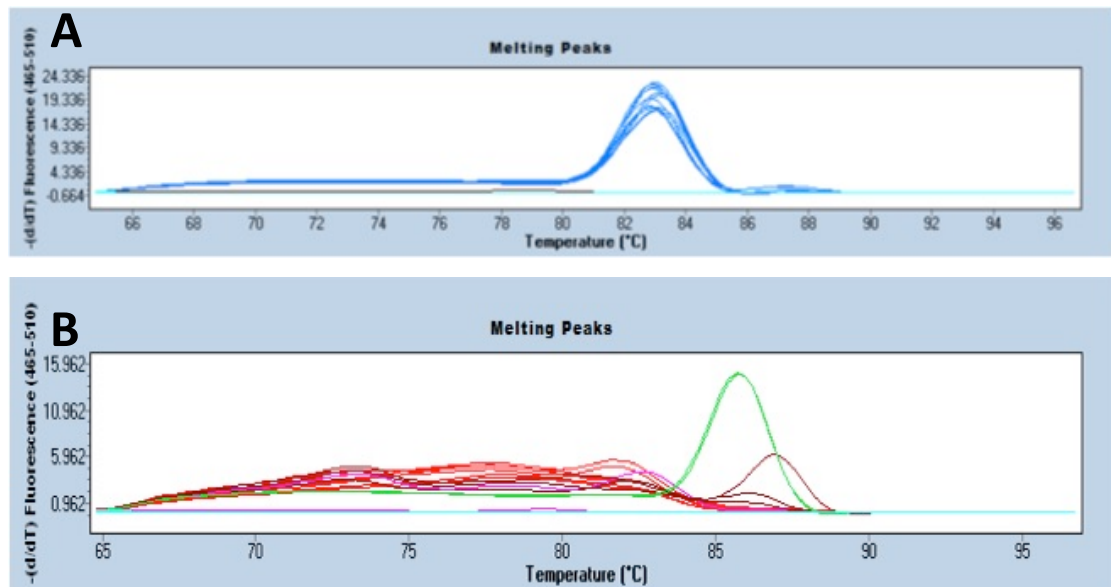


Figure 2.8: Example of adequate melt curve with single peak (A) and an unspecific melt curve with multiple peaks indicating failure of primer and exclusion of that gene in analysis (B).

For stability testing, Ct values for all samples were determined and entered into previously validated software packages geNorm™ (Vandesompele et al., 2002) and Ref Keeper (Xie et al., 2012). RefKeeper is an online tool using a combination of BestKeeper (Pfaffl et al., 2004), Δ Ct method (Silver et al., 2006), NormFinder (Andersen et al., 2004) and geNorm™. All packages result in a method specific stability value based on a unique algorithm where lower numbers indicate higher stability and thus suitability as a reference gene.

Cumulus cells

One of the six samples selected for reference gene stability testing showed no expression for two of six genes tested and therefore was excluded from analysis. Ct values were first entered into geNorm™ alone showing that *UBC* and *RPL32* were the most stable of the genes tested. However geNorm M values were > 1 as determined by the average pairwise variation of each gene against all other genes (< 0.5 indicates adequate stability, $0.5-1$ indicates moderate stability and > 1 indicates low stability; [Vandesompele et al., 2002]; Figure 2.9). Additionally, they showed high variability, as all combinations of genes yielded geNorm V values > 0.15 (as determined by calculating the variability of sequential normalisation factors [$V_{n/n+1}$]; Vandesompele et al., 2002; Figure 2.10), this software “could not determine an optimum number of reference genes to use” and showed no benefit of combining multiple genes to accurately normalise the subsequent data.

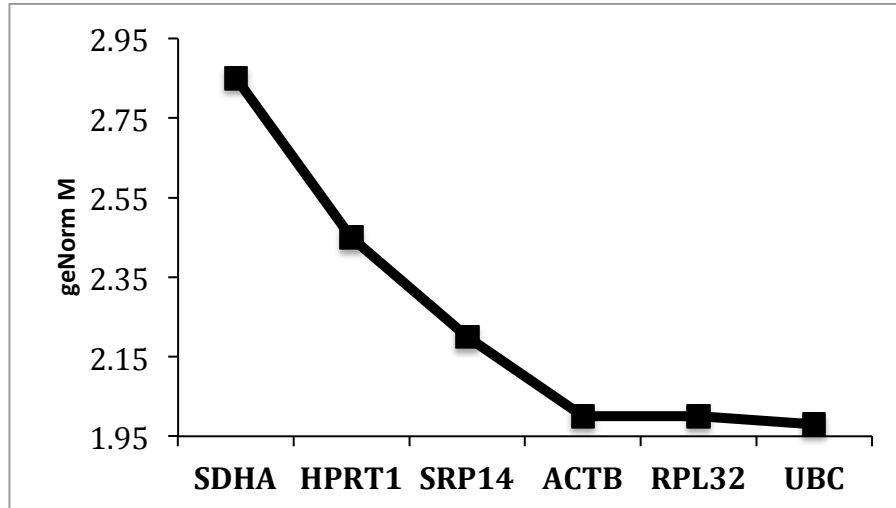


Figure 2.9: Average expression stability (geNorm M) for reference genes tested in cumulus cells. Genes increase in stability from left to right.

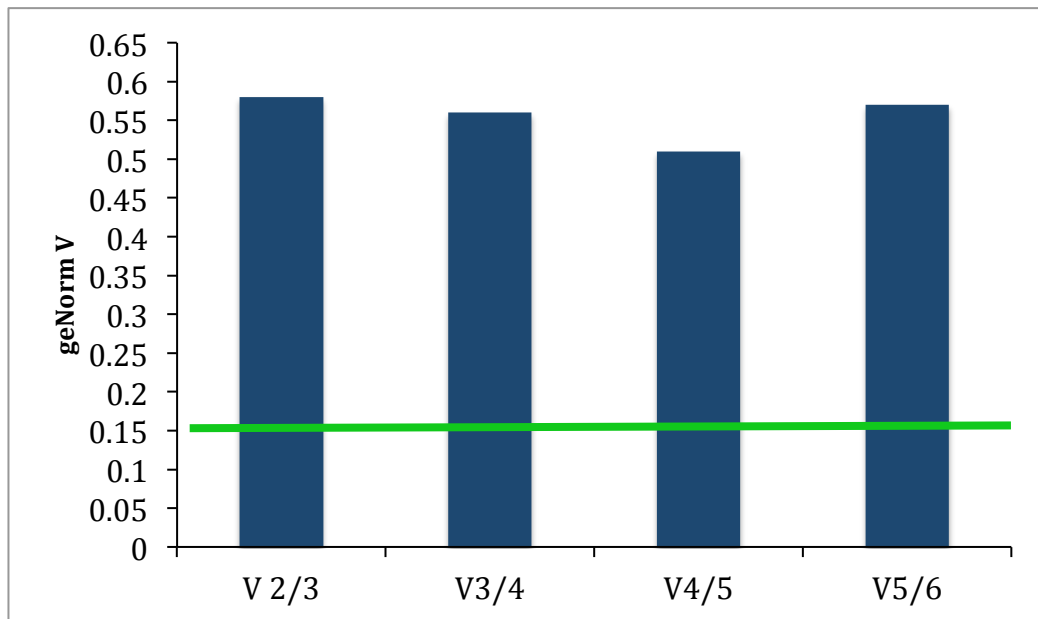


Figure 2.10: Determination of the optimal number of reference genes in cumulus cells. Each bar represents the stability measure (geNorm V value) of combining either 2/3, 3/4, 4/5, or 5/6 reference genes. Green line depicts geNorm V cut off (0.15).

As geNorm did not determine a suitable combination of reference genes, Ct values were then entered into the online combination software RefKeeper to determine the stability across four different validated reference gene selection platforms (Table 2.5). *RPL32* came out repeatedly as the most stable, with *UBC* in second place. Both were chosen for further testing during target gene qPCR.

Rank	RefKeeper	Δ Ct method	BestKeeper	NormFinder	geNorm
1	<i>RPL32</i> 1	<i>RPL32</i> 1.95	<i>RPL32</i> 1.95	<i>RPL32</i> 0.62	<i>RPL32/UBC</i> 1.95
2	<i>UBC</i> 2	<i>UBC</i> 2.62	<i>SRP14</i> 2.14	<i>UBC</i> 1.56	<i>ACTB</i> 1.95
3	<i>SRP14</i> 3.13	<i>SRP14</i> 2.65	<i>ACTB</i> 2.38	<i>ACTB</i> 1.56	<i>SRP14</i> 2.18

Table 2.5: RefKeeper results for cumulus cells. Chosen genes depicted in bold. RefKeeper stability measure derived from combining stability measures from the other four methods (Δ Ct method, Bestkeeper, Normfinder, geNorm). The lower the value the more stable the gene.

Oocytes

Two of six gene primers tested (SDHA and ACTB) showed non-specific melt curves so were excluded from analysis. Ct values were first entered into geNorm™ alone showing that UBC and RPL32 were the most stable of the genes tested, however with geNorm M values > 0.5 (Figure 2.11) they showed medium stability and as all combinations of genes yielded geNorm V values > 0.15 (Figure 2.12), this software “could not determine an optimum number of reference genes to use” and showed no benefit of combining any of the genes to accurately normalise the subsequent data.

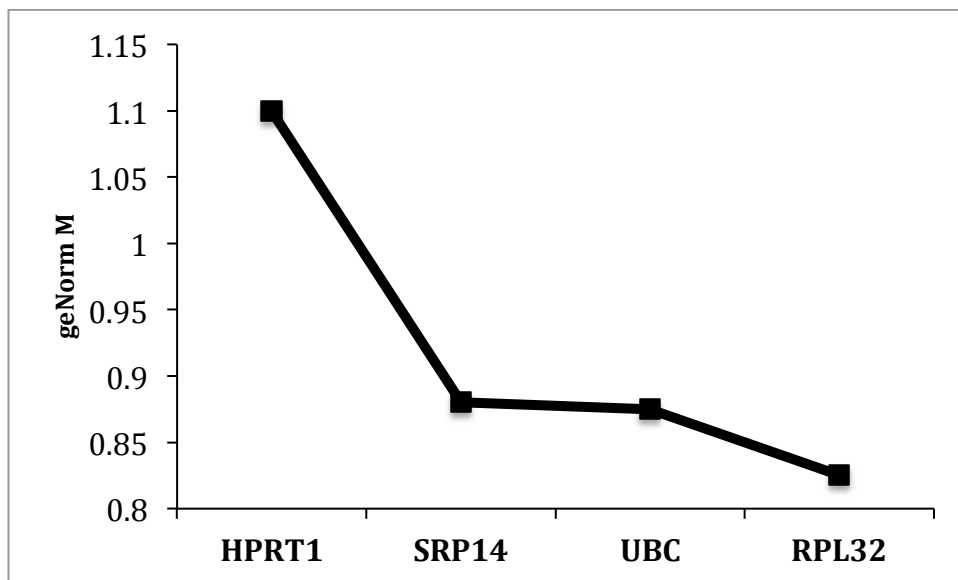


Figure 2.11: Average expression stability (geNorm M) for reference genes tested in single oocytes. Genes increase in stability from left to right.

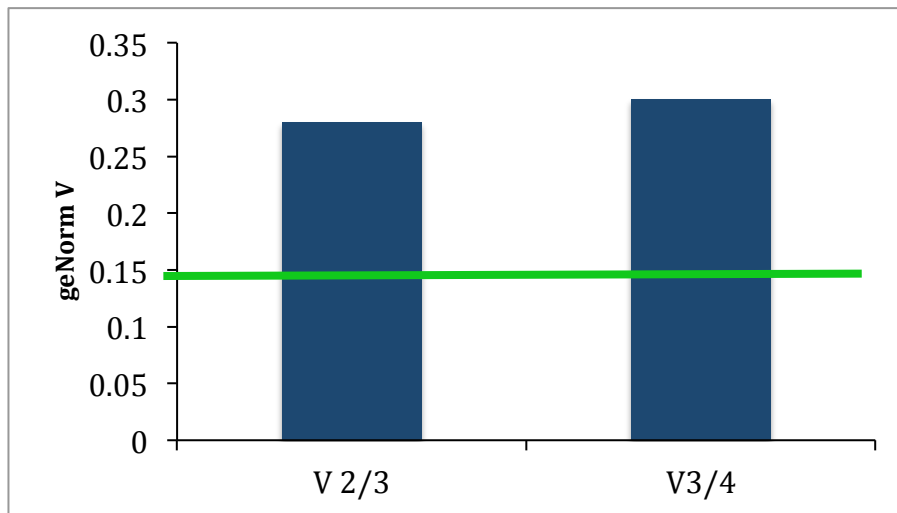


Figure 2.12: Determination of the optimal number of reference genes in single oocytes. Each bar represents the stability measure (geNorm V value) of combining either 2/3 or 3/4 reference genes. Green line depicts geNorm V cut off (0.15).

As for the cumulus samples, Ct values were then entered into the online combination software RefKeeper (Table 2.6). RPL32 and UBC again came out as the most stable and were chosen for further testing during target gene qPCR.

Rank	Refkeeper	Delta ct	Best keeper	Norm finder	geNorm
1	RPL32 1.32	RPL32 0.96	UBC 0.75	RPL32 0.43	UBC/RPL32 0.79
2	UBC 1.73	SRP14 1.02	SRP14 1.02	SRP14 0.56	SRP14 0.89
3	SRP14 2.2	UBC 1.1	RPL32 1.16	UBC 0.85	HPRT1 1.10

Table 2.6: RefKeeper results for oocyte samples. Chosen genes depicted in bold. RefKeeper stability measure derived from combining stability measures from the other four methods (Δ Ct method, Bestkeeper, Normfinder, geNorm). The lower the number the more stable the gene.

Blastocysts

The same two gene primers that failed in the oocyte samples also failed in the blastocysts (*ACTB* and *SDHA*) therefore they were not further considered as reference genes. Additionally one sample did not show normal amplification for any of the reference genes tested and was therefore excluded from analysis. Ct values were first entered into geNorm™ showing that *RPL32* and *SRP14* were the most stable of the genes tested, however with geNorm M values > 0.5 (Figure 2.13) they showed medium stability and as all combinations of genes yielded geNorm V values > 0.15 (Figure 2.14), this software did not give an optimum number of reference genes to use.

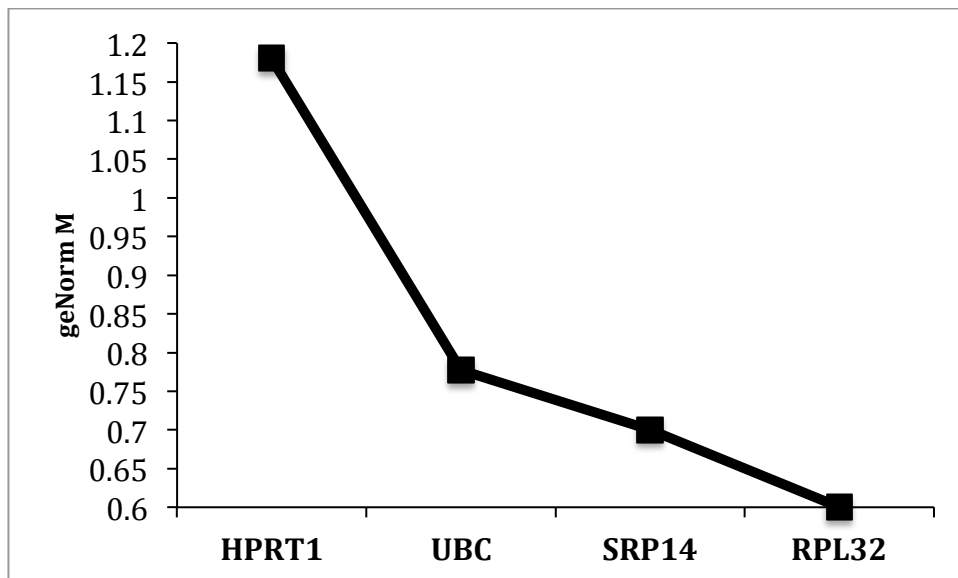


Figure 2.13: Average expression stability (geNorm M) for reference genes tested in blastocysts. Genes increase in stability from left to right

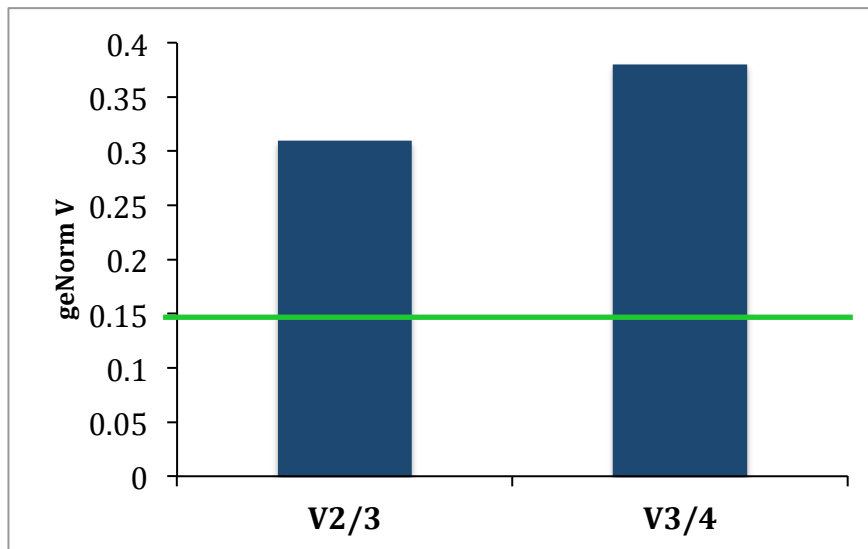


Figure 2.14: Determination of the optimal number of reference genes in blastocysts. Each bar represents the stability measure (geNorm V value) of combining either 2/3 or 3/4 reference genes. Green line depicts geNorm V cut off (0.15).

Ct values were then entered into the online combination software RefKeeper to determine the stability across four different validated reference gene selection platforms (Table 2.7). *RPL32* came out repeatedly as the most stable, with *SRP14* in second place. Both were chosen for further testing during target gene qPCR.

Rank	RefKeeper	Δ Ct method	BestKeeper	NormFinder	geNorm
1	<i>RPL 32</i> 1.19	<i>RPL32</i> 0.94	<i>HPRT1</i> 1.56	<i>RPL32</i> 0.13	<i>RPL32/SRP14</i> 0.48
2	<i>SRP14</i> 2.45	<i>UBC</i> 0.99	<i>RPL32</i> 1.83	<i>UBC</i> 0.37	<i>UBC</i> 0.78
3	<i>UBC</i> 2.45	<i>SRP14</i> 1.21	<i>UBC</i> 1.93	<i>SRP14</i> 1.07	<i>HPRT1</i> 1.8

Table 2.7: RefKeeper results for blastocyst samples. Chosen genes depicted in bold. RefKeeper stability measure derived from combining stability measures from the other four methods (Δ Ct method, Bestkeeper, Normfinder, geNorm). The lower the number the more stable the gene.

2.6 Data analysis

Enzyme linked assays

The primary outcome variables were glucose consumption, lactate and pyruvate production (expressed as pmol/ngDNA/hour). Ratio of Lactate : Glucose was calculated by dividing lactate production by glucose consumption. Glycolytic index was calculated as the percentage of glucose accounted for by lactate production; where two molecules of lactate are produced for every one molecule of glucose is 100%. Data were scrutinised for erroneous values and outliers excluded from subsequent analyses if deemed biologically improbable, most likely due to technical error in one of the assays. Criteria used for cleaning and exclusion of data were as follows; Ratio lactate : glucose > 10 and > 100 pmol/ng DNA/hour for either glucose consumption or lactate production. If this occurred in any COC at any time point all data for that COC were excluded from statistical analyses. Significance was set at $p < 0.05$.

Oxygen consumption rate measurement

The third reading was taken for all basal OCR readings and the reading representing maximal response was used for all inhibitor OCR readings. Data were expressed as pmolO₂/COC/hour or pmolO₂/ngDNA/hour. Data were excluded from subsequent analyses if the Seahorse instrument produced a biologically improbable value. Spare capacity and proton leak were corrected to zero if a negative value was obtained. Percentage data were first arcsine transformed before performing statistical tests.

The word 'replicate' was used in all studies to describe all COCs collected from the abattoir on a single day. The average recovery rate was 3 COCs/ovary i.e. 6 COCs per mare. The range of number of mares represented in each replicate was therefore 6-13. N numbers pertain to number of COCs ($n = 1 = \text{COC}$).

Chapter 3: Characterising equine carbohydrate metabolism and oxygen consumption of equine cumulus-oocyte complexes during *in vitro* maturation.

3.1 Introduction

Production of offspring using *in vitro* technologies remains inefficient in all species. In the horse, collection of immature oocytes and subsequent *in vitro* maturation (IVM) is relied upon to produce offspring via intracytoplasmic sperm injection (ICSI). As outlined in Section 1.4.3, little work has been done to optimize the IVM process in the horse and there are major knowledge gaps concerning the species-specific requirements of the cumulus oocyte complex (COC) during IVM. With increasing knowledge of potential downstream effects on offspring as a result of an inadequate *in vitro* environment during *in vitro* embryo production (IVP) (El Hajj and Haaf, 2013; Fernandez-Gonzalez et al., 2007; Watkins et al., 2007), it is a priority to develop a species-tailored system that is as physiological as possible.

One approach used widely to optimise IVP in other species is to characterise the energy metabolism of the COC. This approach can inform the design of a species-specific maturation medium by defining the major nutrients required by the relevant metabolic pathways and the appropriate concentrations of these nutrients. This has already been carried out for oocytes of the pig, mouse, human, cow, monkey, cat, sheep and rabbit (For review; Krisher et al., 2007). While many of these characterisations have been performed on the denuded oocyte, for the purpose of the studies in this thesis, the intact COC was examined given the interdependent nature in which the oocyte and cumulus cells co-exist, as described in Section 1.3.1.

The measurement of COC metabolism in a meaningful way is technically challenging, as the heterogeneity in developmental potential of COCs ideally necessitates assessment of individual complexes, to define the metabolism corresponding to optimum oocyte competence. This requires highly sensitive assays. The relative metabolic quiescence of single oocytes and COCs means that

‘analysis media’ with lower substrate concentrations have often been used in order to permit detection of consumption/accumulation of substrates (Hardy *et al.*, 1989; Sturmey and Leese, 2003). As the *in vitro* environment is an already ‘imperfect model’ in which to study metabolism (Section 1.5.3), attempts were made in the current studies to minimise analysis-related changes by using only maturation media proven to be capable of producing developmentally competent oocytes (M199 with Earle’s salts, 10% FBS, 25 µg/ml gentamicin with 5 mU/ml FSH; Hinrichs *et al.*, 2014, 2005). However, using this medium presented challenges due to the glucose concentration (5.6 mM) and presence of serum, both of which have the potential to confound the use of standard substrate assay systems by not allowing small changes in substrate depletion to be detected. To overcome these challenges, the selected protocol had to enable reliable and repeatable detection of differences in substrate concentrations between media samples and reference droplets post-incubation.

Information about oxidative metabolism is best acquired by measuring the oxygen consumption rate (OCR) (Brand and Nicholls, 2011). Mitochondrial integrity of the oocyte is crucial to successful fertilisation and embryo development (Dumollard *et al.*, 2007). All mitochondria in the embryo are maternally inherited and there is little or no further replication of mitochondria until the blastocyst stage (recently defined in the horse; Hendriks *et al.*, 2018), such that damage incurred during oocyte maturation has the potential to impair not only their function but also the transfer of healthy mtDNA to the next generation (Smeets, 2013). Mitochondrial DNA (mtDNA) is especially susceptible to damage, given the local production of reactive oxygen species (ROS) and limited mtDNA repair mechanisms (Van Blerkom, 2011).

Most studies evaluating mitochondrial function in the oocyte have thus far measured mitochondrial potential by relative fluorescence staining, assessing mt copy number, mitochondrial localisation and cellular ATP content (Van Blerkom, 2011). In the horse, the use of MitoTracker staining has shown that mitochondrial membrane potential increases throughout maturation, and that the aggregation pattern becomes more granular and heterogeneous (Ambruosi *et al.*, 2009; Caillaud

et al., 2005; Torner et al., 2007). Additionally, when mtDNA copy number was analysed before and after IVM, it was found to decrease only in aged mares (> 12 years) and not in young (Rambags et al., 2014, 2006).

While the above methods provide valuable information on mitochondrial function, more recent technology based on measurement of OCR before and after the use of respiratory inhibitors allows detailed assessment of individual elements of the electron transport chain and thus mitochondrial efficiency. There are numerous reports in somatic cells, including cancer cells, and more recently in embryos (Houghton et al., 1996; Manes and Lai, 1995; Trimarchi et al., 2000); however, there are only two studies to date utilising these methods in mammalian oocytes (Obeidat et al., 2018; Sugimura et al., 2012). The use of respiratory inhibitors such as oligomycin (ATP synthase inhibitor), FCCP (uncoupler of the inner mitochondrial membrane) and rotenone and antimycin (inhibitors of complex I and III respectively) allow estimation of respiration coupled to ATP production, OCR attributable to proton leak, non-mitochondrial respiration, and spare respiratory capacity. These measures can build a detailed picture of mitochondrial health and also aid in estimating relative contribution of OXPHOS and glycolysis to overall ATP production (for detailed explanation of these elements, see Section 1.5.2).

In addition to informing and optimising IVM protocols, a further driver to study and characterise the metabolism of the COC is the desire to identify biomarkers of viability and to subsequently design non-invasive methods for determination of individual oocyte developmental competence. While this has been attempted in various species with promising results, a reliable predictive marker remains elusive.

Summary

The aim of these studies was to characterise the glycolytic and oxidative metabolism of the equine COC during *in vitro* maturation, describe mitochondrial function and efficiency throughout maturation and discover whether differences in metabolism were associated with factors known to influence development including meiotic status and cumulus classification. The final aim was to discover if glucose metabolism during IVM had predictive value for developmental competence.

3.2 Objectives

1. Determine glucose consumption and lactate production of equine COCs during IVM and explore potential relationships between these nutritional markers and:
 - a) Cumulus classification and oocyte maturation status (Pilot study 1).
 - b) Markers of developmental competence including subsequent embryo cleavage and blastocyst development (Study 3.1).
2. Quantify the temporal changes in glucose/lactate metabolism during different phases of IVM, (0-10 hours, 10-20 hours, 20-30 hours) (Pilot study 2).
3. Evaluate OCR in intact COCs over the time course of IVM and explore associations between OCR and glucose/lactate metabolism (Study 3.2).
4. Evaluate mitochondrial function over the time course of IVM (Study 3.3).

3.3 Materials and methods

The study design is outlined in Figure 3.1 and individual elements are discussed below when they differ from descriptions in Chapter 2.

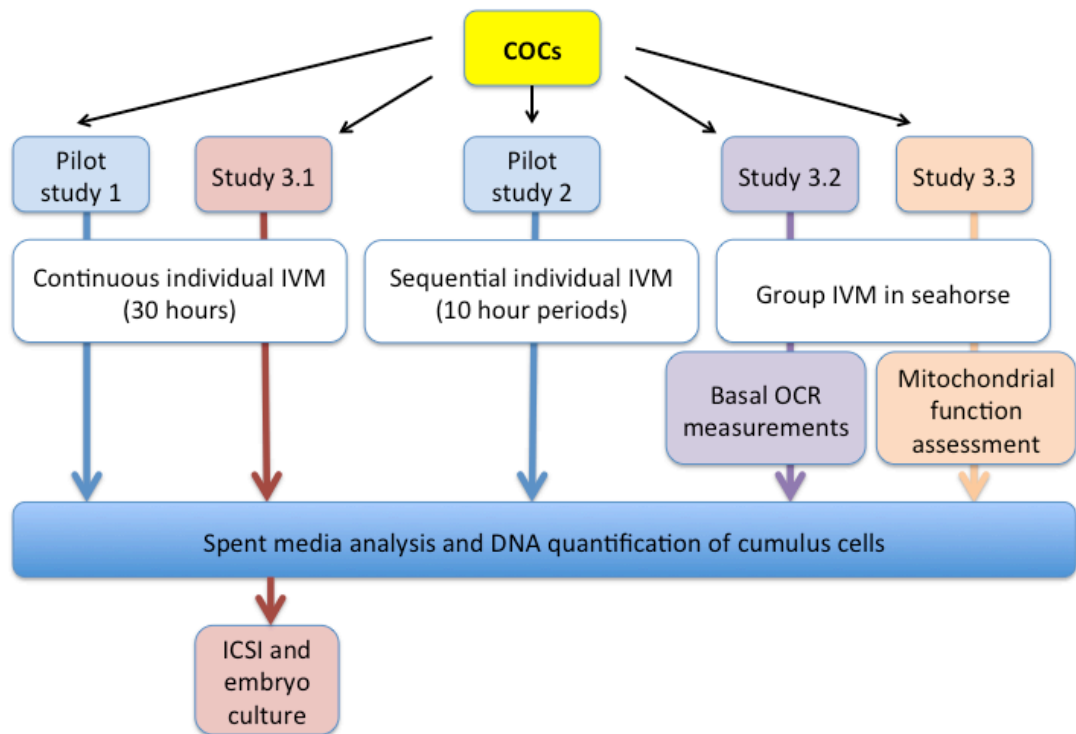


Figure 3.1: Schematic flow diagram of study design. Abbreviations used are as follows: COCs = cumulus oocyte complexes, IVM = in vitro maturation, OCR = oxygen consumption rates, ICSI = intra cytoplasmic sperm injection.

General methods

To meet the study objectives, COCs were recovered from abattoir-derived ovaries, classified as compact (Cp), expanded (Ex) or corona radiata-only, and held overnight as previously described in Section 2.1. Maturation was performed in Maturation medium (M199 with Earle's salts, 10% FBS, 25 µg/ml gentamicin with 5 mU/ml FSH) at 5% CO₂ in air. IVM conditions varied slightly between studies as outlined below.

Pilot Study 1

Maturation was carried out in individual 10- μ l droplets under oil, with nine droplets per dish. Only Ex and Cp COCs were used. Three additional medium droplets in each dish remained empty and were used as reference droplets. Dishes were continuously incubated for the entire IVM period (30 hours; Figure 3.1; See Section 2.2.4 for full description). After the maturation period was completed, COCs were denuded and classified as Metaphase II, intact or degenerated as previously described in Section 2.1.4. All denuded cumulus cells from each COC were placed in a labelled 96-well plate and kept at -20° C for later DNA quantification analysis. Spent media samples were frozen at -80° C and analyses of thawed samples carried out using the Consumption and release method (CoRe) as described in Section 2.2

Study 3.1

COCs were individually cultured and denuded, and cumulus cells and spent media analysed, as described for Pilot Study 1. After denuding, metaphase II oocytes underwent ICSI and subsequent embryo culture as described in Section 2.1.6.

Pilot Study 2

COCs were individually cultured and dishes were prepared as described above however each individual COC, was moved every ten hours from one dish to another therefore three dishes were prepared per nine COCs (Figure 3.1; See Section 2.2.4 for full description). The oocytes were denuded and classified, and cumulus cells and spent media were analysed, as described for Pilot Study 1.

Study 3.2

Culture for IVM was carried out in the conditions described above, with COCs cultured in Seahorse plates in 180 μ l of maturation medium (see Section 2.4.3 for full description). To optimize the measurement of changes in oxygen tension across the study period, COCs were cultured in groups of three of the same cumulus type (Cp or Ex), whereas corona radiata-only oocytes were cultured in groups of six. The Seahorse plate was removed from the incubator and placed in the Seahorse XFp analyser at three different time points (four, twelve and twenty-eight hours after

the start of IVM; IVM + 4hours, IVM + 12 hours and IVM + 28 hours). Three basal OCR readings were obtained (pmolO₂/well/min) at each time point. After each set of readings was complete the plate was replaced in the incubator. After 30 hours, COCs were placed directly in a plate without stripping and frozen for later DNA analysis. Spent medium was collected directly from the Seahorse plate well and media analyses were carried out as for the previous studies.

Study 3.3

Culture for IVM was carried out directly in Seahorse plates as for Study 3.2. Separate plates were prepared for the two analysis time points to be performed. Mitochondrial function was evaluated at either IVM + 4 hours or IVM + 28 hours. To do this, after calibration, three basal oxygen consumption rate readings (pmolO₂/well/min), were recorded before a series of respiratory inhibitors were sequentially added to each well; 1) Oligomycin [1 µM] 2) FCCP [5 µM] 3) Antimycin/Rotenone [2.5 µM]. *See Section 2.4.3 for full description of method. After the readings were obtained, COCs were placed into a plate and frozen for DNA quantification. Spent media analysis was not performed.

Statistical analysis

In Pilot Studies and Study 3.1, the primary outcome variables were glucose consumption, lactate and pyruvate production (expressed as pmol/ngDNA/hour). Univariable analyses using the Student's t-test test were performed to explore associations between substrate consumption and maturation status and between substrate consumption and cumulus classification. Linear regression models were used to explore associations between substrates and between substrate consumption/production and DNA content. Significance was set at $p < 0.05$.

In Studies 3.2 and 3.3, the third basal OCR measurement obtained was used to represent basal OCR, and the measurement representing maximal response was used as the inhibitor-specific OCR reading. Data were expressed as pmolO₂/COC/hour or pmolO₂/ngDNA/hour. One-way ANOVA was used to explore associations between OCR and time points and between OCR and cumulus classification, while

linear regression was used to explore associations between OCR and glucose/lactate measurements. For full details on statistical analyses including criteria for data cleaning and replicates see Section 2.5.

3.4 Results

3.4.1 Pilot study 1: Glucose consumption and lactate production over the 30 hours of IVM

Twenty-nine COCs, obtained and processed in a single replicate, were evaluated. Glucose consumption rate (mean 1223 and range, 300- 1965 pmol/COC/hour) and lactate production rate (mean 1851 and range, 732- 2909.5 pmol/COC/hour) were positively correlated to each other ($p < 0.01$) and to the DNA content of the COCs ($p < 0.01$; Figure 3.2). Almost 25% of COCs (7/29) depleted all available glucose from the 10- μ l droplets of 5.6 mM glucose, M199-based media over the 30 hours of IVM (total consumption ~ 55980 pmol). Those COCs which depleted all available glucose had a reduced glycolytic index (100% is the formation of 2 lactate molecules for each glucose consumed); only 60% of glucose was accounted for by lactate production vs. 95% of glucose being accounted for by lactate production in those COCs for which glucose did not become limiting ($p = 0.02$). There was no difference in glucose and lactate metabolism between COCs containing a MII oocyte and those containing a degenerated oocyte at the end of IVM. COCs with compact (Cp) and expanded (Ex) cumulus differed in DNA content (125 ng/COC vs. 79 ng/COC respectively, $p < 0.001$; overall mean 107.26 ng/COC). Numerically, Cp COCs had higher glucose consumption and lactate production, however when normalised for DNA content this difference disappeared (Figure 3.3).

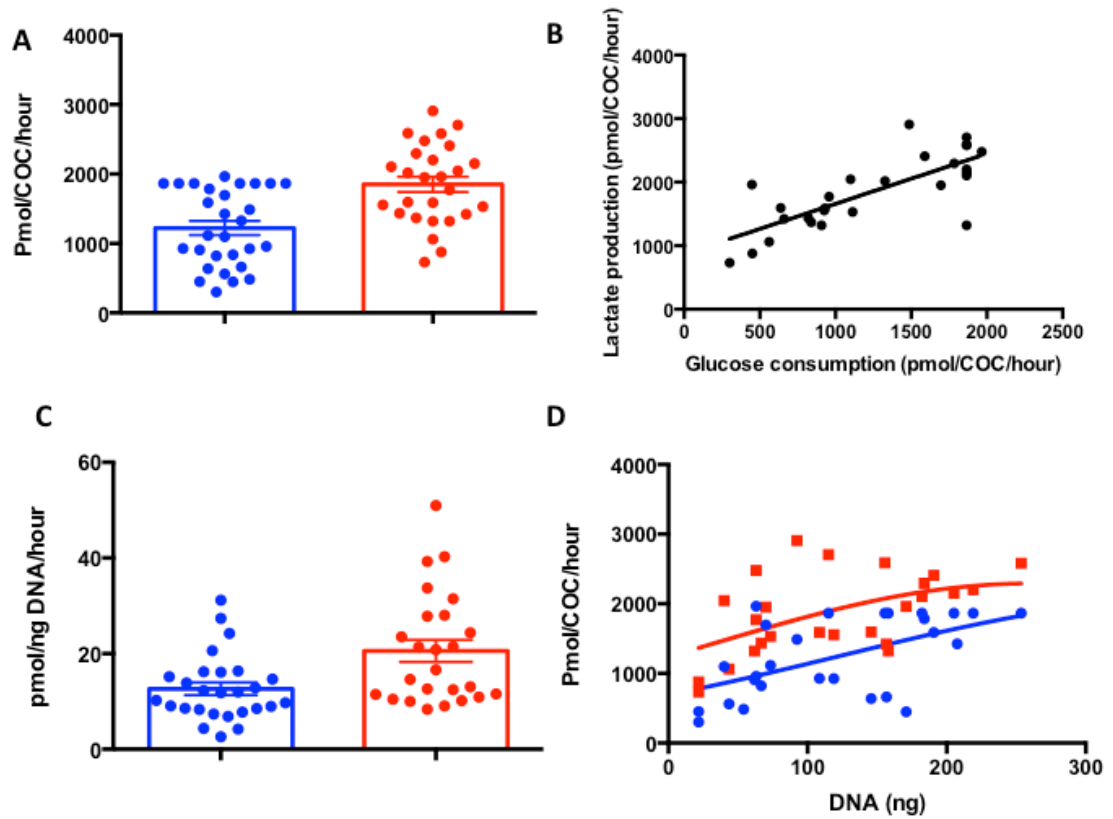


Figure 3.2: Data describing glucose consumption (BLUE) and lactate production rates (RED) in equine COCs during in vitro maturation (n = 29). Panels A & C denote the mean (\pm SEM) rates for individual COC and are presented per COC/Hour (A) and per ng DNA/hour (C). Panel B shows linear correlation between glucose consumption and lactate production rates ($r^2 = 0.59$; $p < 0.001$). Panel D shows correlation of both glucose consumption and lactate production with DNA content ($r^2 = 0.3$; $P = 0.01$).

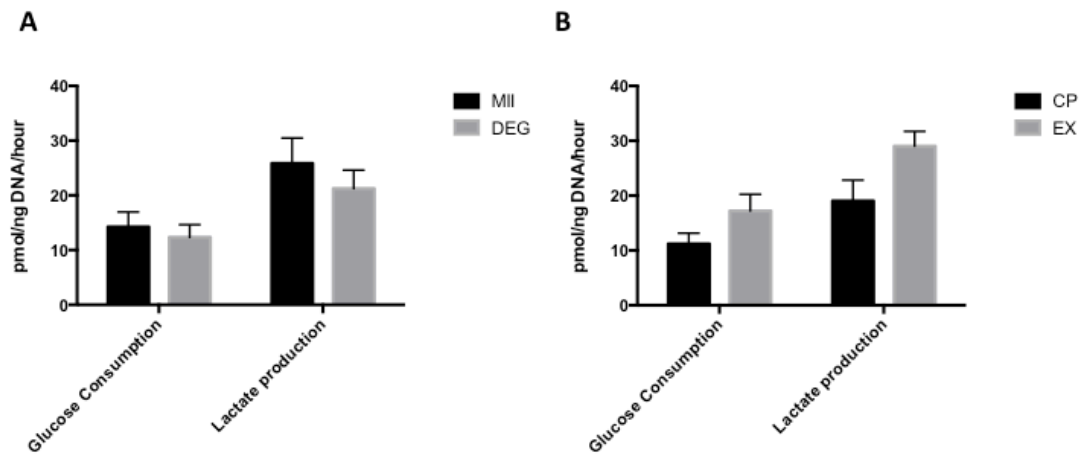


Figure 3.3: Glucose consumption and lactate production rates by maturation status of the oocyte at the end of the culture period (A; metaphase II [MII] and degenerated [DEG] oocytes) and by cumulus classification (B; compact [Cp] and expanded [Ex]). Bars represent mean \pm SEM. ($n = 29$). There were no statistically significant differences in metabolic indices between either maturation status or cumulus classification.

3.4.2 Study 3.1: Association of Glucose/Lactate/Pyruvate metabolism and developmental markers after ICSI.

Eighty-eight COCs were analysed over five replicates.³ Fifty-five oocytes reached metaphase II (63% maturation rate). Forty-nine of the 55 MII oocytes subsequently underwent ICSI and embryo culture; 6 oocytes were either used for other studies or were lost due to technical issues. The cleavage rate at 48 hours was 75.5% (37/49), and 4 blastocysts were produced (8.2%).

Mean glucose consumption and lactate production rates were only evaluated in MII oocytes (n = 50) and the resulting data were similar to those obtained during the pilot study (glucose consumption rates 173.4 to 1866 pmol/COC/hour; lactate production rates, 272.9 to 2683.5 pmol/COC/hour). Pyruvate concentrations were also measured and found to increase over the course of IVM, indicating production and secretion of pyruvate by the COCs (0- 117 pmol/COC/hour, Table 3.1).

Eighteen per cent (9/50) of MII COCs depleted all available glucose from the droplet. As in Pilot Study 3.1, these oocytes had a decreased glycolytic index compared to those for which glucose did not become limiting, and they also produced less pyruvate (0.23 vs. 0.73 pmol/ng DNA/hour; p = 0.01; Table 3.1). It was noteworthy that while numbers were small, depletion of available glucose during IVM did not appear to affect either cleavage rate or blastocyst rate (66.7% cleavage and 11% blastocysts, vs. 77.5% and 7.5%, respectively, for oocytes that did not deplete glucose during IVM).

³ Data derived from control group in Experiment 5.1 (Chapter 5).

	Depleted (n = 9)	Undepleted (n = 41)	P
Glucose consumption rate (pmol/ng DNA/hour)	12.67 ± 0.97	9.99 ± 1.11	0.3
Lactate production rate (pmol/ng DNA/hour)	15.06 ± 1.56	18.39 ± 1.82	0.4
Pyruvate production rate (pmol/ng DNA/hour)	0.23 ± 0.08	0.73 ± 0.09	0.01*
Lactate: glucose ratio	1.17 ± 0.07	2.12 ± 0.17	0.01*
Cleavage rate	66.7% (6/9)	77.5% (31/40)	0.5
Blastocyst rate	11.1% (1/9)	7.5% (3/40)	0.6

Table 3.1: Mean (\pm SEM) substrate consumption and production rates and developmental markers (cleavage and blastocyst rate) for Metaphase II COCs that depleted all available glucose during IVM and those that did not. * denotes significance.

In those MII COCs for which glucose was not limiting, there was no association between glucose consumption, lactate production, pyruvate production rates or lactate : glucose ratio during IVM and cleavage rate or blastocyst rate after ICSI ($p > 0.05$; Figure 3.4).

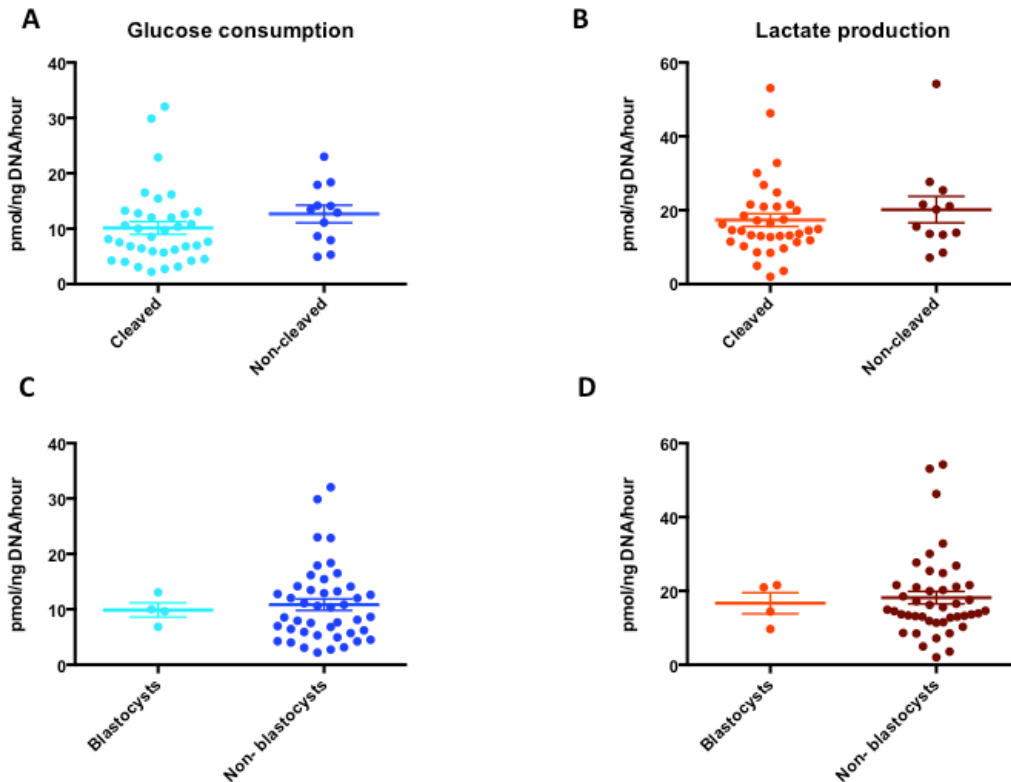


Figure 3.4: Individual COC values for glucose consumption rates (BLUES; Panel A and C) and lactate production rates (REDS; Panels B and D) during IVM plotted around the mean \pm SEM ($n = 49$ injected oocytes). Cleaved vs. un-cleaved oocytes at 48 hours are compared (A-B) and blastocysts vs. non-blastocyst compared (C-D). No statistically significant differences were found between groups.

3.4.3 Pilot study 2: Temporal changes in glucose consumption and lactate production rates during IVM

Eighteen COCs were evaluated, of which three were excluded during data cleaning leaving 15 COCs for statistical analyses. Glucose consumption and lactate production rates decreased significantly during the final period of IVM (20-30 hours) compared to 0-10 and 10-20 hours (Figure 3.5). The lactate : glucose ratio did not vary with time (mean 2.37 ± 0.3). No COCs depleted all available glucose during any of the ten-hour analysis segments.

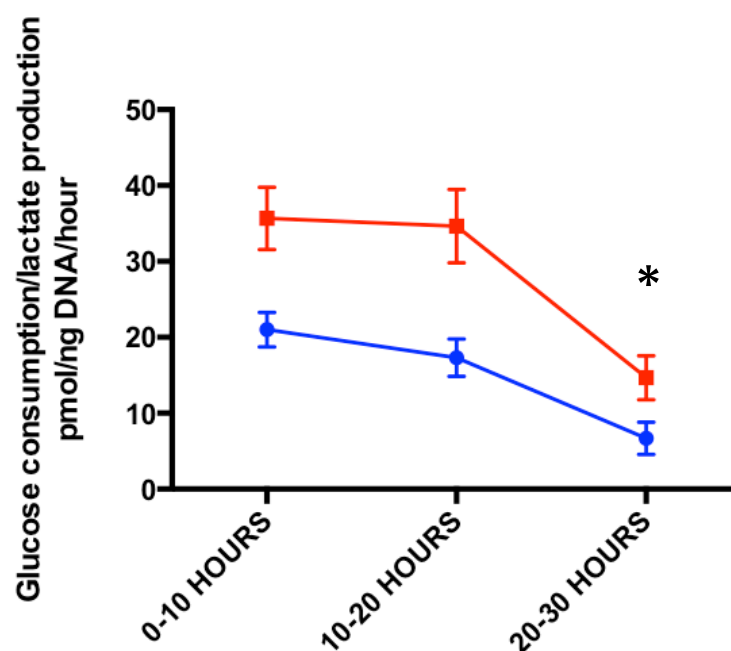


Figure 3.5: Temporal changes in glucose consumption (BLUE) and lactate production (RED) rates during IVM ($n = 15$). Data are presented as mean \pm SEM. * denotes significant difference at 20-30 hours for both substrates ($p < 0.01$).

3.4.4 Study 3.2: Basal oxygen consumption rate over the time course of IVM and associations with glucose and lactate metabolism.

Fifty-one COCs were used for this experiment across three replicates. Data from nine COCs were excluded from subsequent analysis due to biologically implausible OCR values from the Seahorse instrument, and in five of the wells ($n = 3$ COCs per well), one COC was not located at the end of the experiment and accurate DNA quantification could not therefore be performed.

Oxygen consumption rates (OCR) varied widely (IVM + 4 hours; range 370-4875 pmol/COC/hour; IVM + 12 hours; range 259-6176 pmol/COC/hour; IVM + 28 hours; range 303-5805 pmol/COC/ hour). There were differences between values derived for COCs within different cumulus categories (Figure 3.6), however, these differences were not statistically significant, and were lost when individual COC OCRs were standardised to account for differences in COC DNA content. On this basis, DNA-corrected COC OCR data were pooled across morphological classifications before carrying out further analyses. DNA content tended to be positively correlated with OCR at all three time points ($r^2 \geq 0.3$; $p = 0.2$; Figure 3.6C). Basal OCR did not vary with time (IVM + 4hours, IVM + 12hours and IVM + 28hours; Figure 3.6)

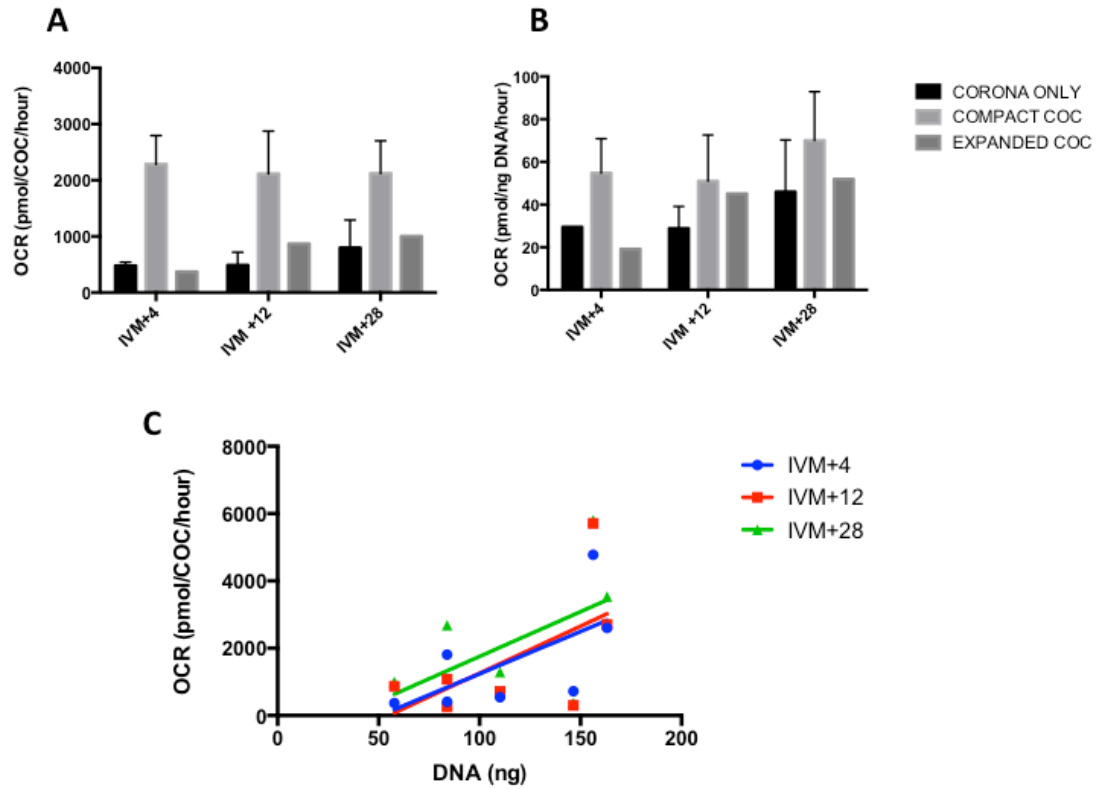


Figure 3.6: Panel A and B: Mean (\pm SEM) Basal COC oxygen consumption rates (OCR) measured at 4, 12 and 28 hours after the initiation of the 30 hour in vitro maturation period (IVM + 4, + 12, + 28) for corona-only COCs ($n = 12$), compact COCs ($n = 27$ Panel A, $n = 12$ Panel B) and expanded COCs ($n = 3$). Panel C: Association between COC DNA content and OCR (all associations $r^2 \geq 0.3$; $p = 0.2$). Each data point represents one Seahorse well ($n = 3$ COCs).

Glucose consumption rates ($r^2 = 0.83$, $p < 0.01$; $r^2 = 0.78$, $p = 0.01$; $r^2 = 0.48$, $p = 0.08$) at IVM + 4 hours, 12 hours and 28 hours respectively; Figure 3.7A) and lactate production rates ($r^2 = 0.76$, $p = 0.01$; $r^2 = 0.73$, $p = 0.02$; $r^2 = 0.5$, $p = 0.06$) at IVM+ 4 hours, 12 hours and 28 hours respectively; Figure 3.7B) were positively correlated with OCR at all time points measured during IVM. Lactate : glucose ratio was not correlated to OCR at any time point ($p > 0.5$ for all time points; Figure 3.7C).

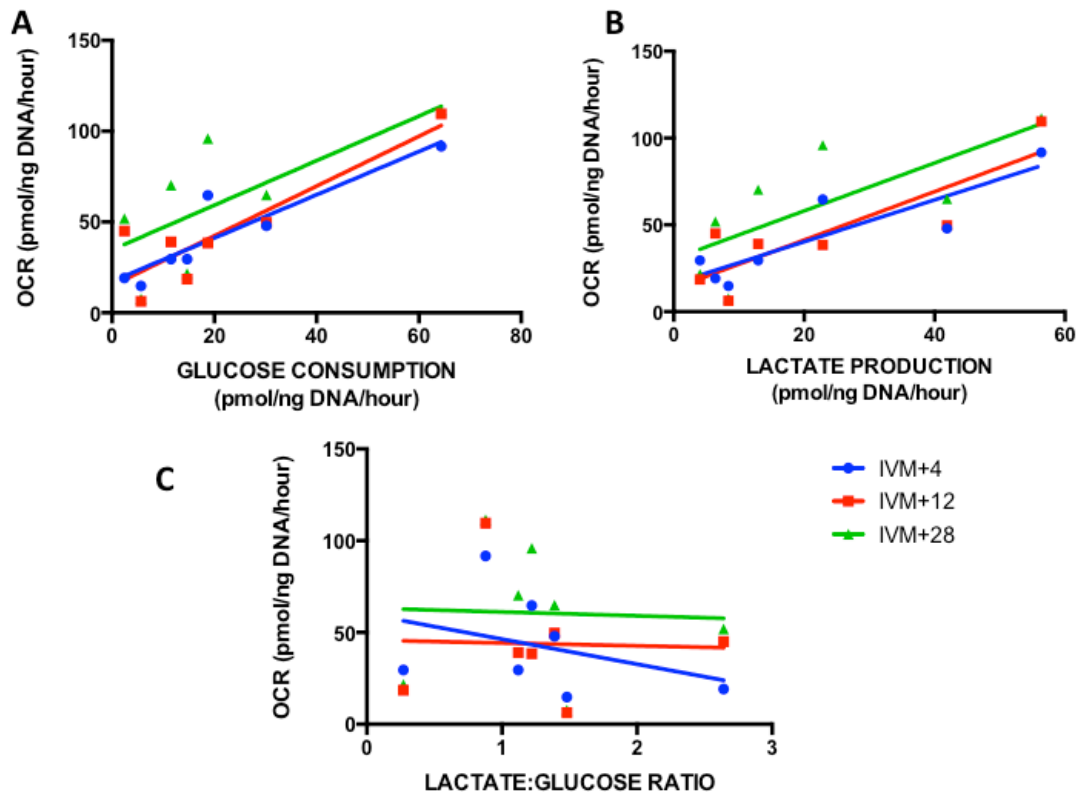


Figure 3.7: Associations between substrate analyses of spent media and oxygen consumption rates (OCR) at 4, 12 and 28 hours after the initiation of IVM (each data point represents one well; $n = 27$). Panel A: Glucose consumption rate and OCR ($r^2 = 0.83$, $p < 0.01$; $r^2 = 0.78$, $p = 0.01$; $r^2 = 0.48$, $p = 0.08$ at IVM + 4 hours, 12 hours and 28 hours respectively). Panel B: Lactate production rate and OCR ($r^2 = 0.76$, $p = 0.01$; $r^2 = 0.73$, $p = 0.02$; $r^2 = 0.5$, $p = 0.06$ at IVM + 4 hours, 12 hours and 28 hours respectively). Panel C: Lactate : Glucose ratio and OCR (association not statistically significant at any time point $p > 0.5$).

3.4.5 Study 3.3: Mitochondrial function over the IVM period.

Sixty-nine COCs were used across three replicates. Data for 12 COCs were excluded from subsequent analysis due to OCR readings from the Seahorse instrument that lacked biological plausibility.

Oxygen consumption rate coupled to ATP production ($47 \pm 10\%$ and $58 \pm 4\%$ at IVM + 4 and 28 hours respectively), non-mitochondrial OCR ($38 \pm 10\%$ and $31 \pm 5\%$ at IVM + 4 and 28 hours respectively) and OCR attributed to proton leak ($17 \pm 6\%$ and $17 \pm 2\%$) did not vary between the outset and end of IVM (Figure 3.9). Spare capacity however, changed significantly during IVM ($85 \pm 31\%$ and $5 \pm 3\%$ at IVM + 4 and 28 hours respectively; $p < 0.01$). In evaluating data from individual wells, 100% responded to FCCP at IVM + 4 hours, demonstrating spare respiratory capacity, as opposed to only 50% of wells responding at IVM + 28 hours (Figure 3.8).

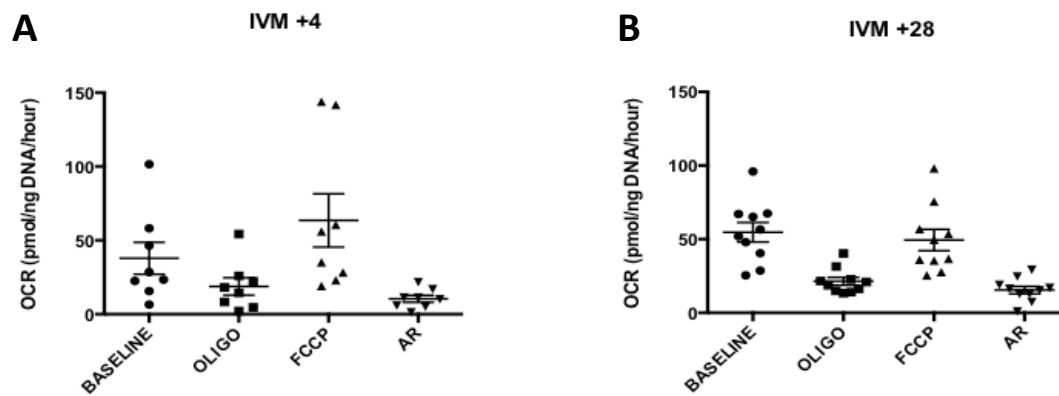


Figure 3.8: Oxygen consumption rates after addition of respiratory inhibitors to evaluate mitochondrial function at IVM + 4 hours (outset) and IVM + 28 hours (end). Panels A and B; Data are presented as OCR normalised for COC DNA content, each data point represents one well, with mean \pm SEM ($n = 57$).

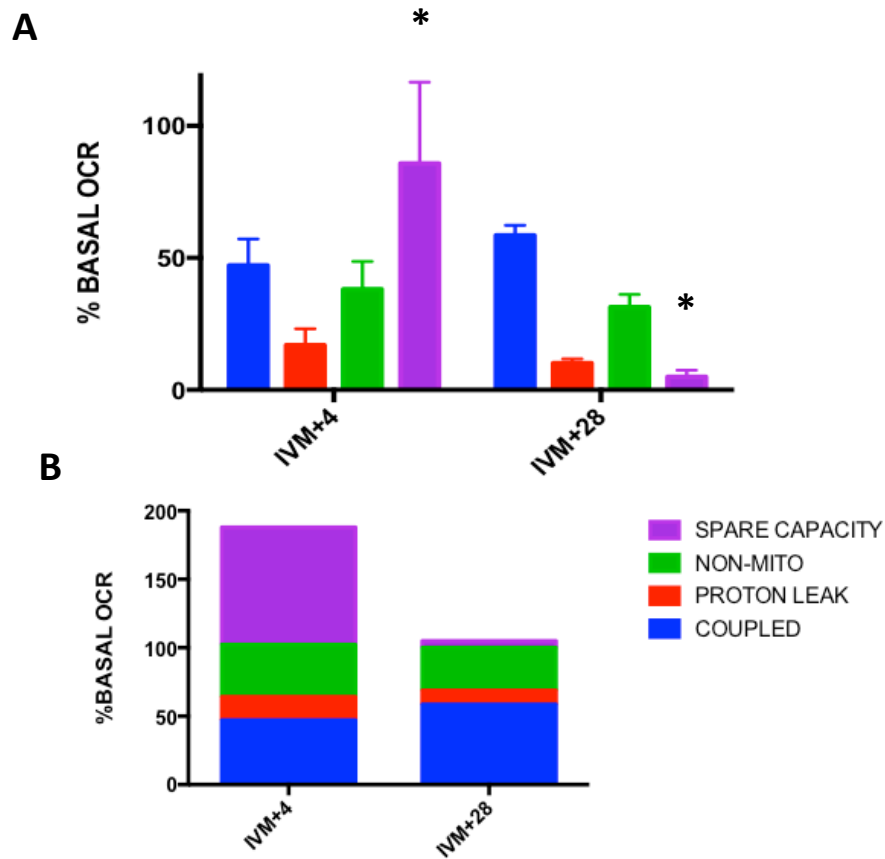


Figure 3.9: Mitochondrial function at IVM + 4 (outset) and IVM + 28 hours (end) as determined from respiratory inhibitor studies (Panel A; $n = 57$). Oxygen consumption rates (OCR) coupled to ATP production (BLUE), OCR associated with proton leak (RED), non-mitochondrial OCR (GREEN) and spare respiratory capacity (PURPLE) are expressed as % basal OCR. Mean \pm SEM are presented in Panel A and mean values are stacked in Panel B. Asterisks represent significance difference between spare capacity at time IVM + 4 and IVM + 28 hours; $P < 0.01$.

3.5 Discussion

This is the first report describing carbohydrate metabolism, oxygen consumption and mitochondrial efficiency in equine COCs. In addition, it is the first time the use of the Seahorse XFp analyser has been reported for COCs in any species. The data demonstrate that glycolysis was the major active pathway of glucose metabolism in equine COCs during IVM, as 95% of glucose consumed was accounted for by lactate production in COCs for which glucose was not limiting. While glycolysis what the main pathway for glucose utilisation however, the high concurrent OCR demonstrates that equine COCs are not “mainly glycolytic” as other species but rather OXPHOS plays a significant role in energy metabolism. Glycolysis is the main pathway reported in previous studies on equine COCs, as well as in those of other species studied to date (Biggers et al., 1967, Gonzalez-Fernandez et al., 2018, Sutton et al., 2003). While OCR was measured in the bovine study confirming that glycolysis was the main pathway in energy provision (Sutton et al., 2003), OCR was not measured in either the murine (Biggers et al., 1967) or equine study (Gonzalez-Fernandez et al., 2018).

Most studies of this nature have been carried out on the denuded oocyte and demonstrated an uptake of pyruvate (Harris et al., 2009), a net production of pyruvate was found in the present study. This has been reported previously in equine and murine COCs during IVM (Downs et al., 2002, Gonzalez-Fernandez et al., 2018, Harris et al., 2007). It was demonstrated as early as 1985 by Leese and Barton that isolated murine cumulus cells were capable of producing pyruvate from both glucose and lactate. It was further shown in the mouse that the pyruvate concentration was higher in the oviduct containing the ovulated COC/cumulus cells than the oviduct without (Gardner and Leese, 1990; Harris et al., 2005). Given that pyruvate is an important energy source for oocytes and embryos immediately post-fertilisation, this observation was unexpected. However, formation and secretion of pyruvate could have two explanations; the cumulus cells produce pyruvate in excess of that needed by the oocyte, perhaps as a result of some metabolic decoupling occurring in the *in vitro* conditions, or more likely as a defence

mechanism against Reactive Oxygen Species (ROS) to aid in redox balance, since pyruvate can act as a ROS scavenger ($\text{Pyruvate} + \text{H}_2\text{O}_2 \rightarrow \text{Acetate} + \text{CO}_2 + \text{H}_2\text{O}$; O'Donnell-Tormey et al., 1987). For example, in somatic cells, pyruvate production and secretion to the culture media is prioritised over intracellular ATP production in order to protect against ROS in the scavenging of H_2O_2 (O'Donnell-Tormey et al., 1987). Interestingly, Downs and Hudson, (2000) showed that in a microdrop environment (8 μl) secreted pyruvate build-up meant that glucose alone as a substrate was sufficient to support meiotic maturation in murine COCs whereas in larger volumes (1 ml) pyruvate addition was required for optimal meiotic maturation rates. For this reason, culture media for somatic cells often includes pyruvate; something that will be explored for equine IVM media in Chapter 4.

Up to 24% of COCs depleted all the available glucose from a 10- μl droplet of M199 based maturation media (5.6 mM glucose) and the glucose consumption rate was positively correlated to the DNA content of the COC. This depletion of available glucose has not been previously reported and suggests that the current IVM environment, as often reported (10 μL medium per COC; Choi et al., 2016) may be inadequate in terms of glucose supply. In other species such as the mouse, human and cow, immature oocyte retrieval is performed via follicular aspiration and therefore COCs are typically collected with only a few layers of cumulus, which could account for the lack of depletion. In two reports, the number of cumulus cells in murine COCs were counted and averaged 2060 per cumulus mass (Leese and Barton, 1985) and 1200 (Preis et al., 2005). Although not counted in this study, as an estimate using DNA content measured in single bovine cumulus cells (6.45pg; Mirsky and Osawa, 1961) the average number of cumulus cells in the current study would be 16,629 per COC. As reported by Sutton et al. (2003) in cattle, normalising results for DNA content allowed for more accurate comparison of metabolic measurements between COCs, given the wide range of COC size both due to number of cells and expansion. When adjusted for DNA content, the glucose consumption and lactate production rates during IVM by equine COCs were approximately half of those reported by Sutton et al., (2003) by bovine COCs (~30

pmol glucose produced/ng DNA/hour and ~ 70 pmol lactate produced/ng DNA hour).

In the single previous report available on glucose metabolism by equine COCs, nuclear magnetic resonance (NMR) spectroscopy was used as the measurement method and results were reported in nmol/COC/entire IVM period (Gonzalez-Fernandez et al., 2018). The COC-to-medium ratio also varied, as COCs were cultured in groups of 1-25 in 250- μ l droplets. Despite these differences in experimental design and analysis, it is interesting that metabolic rates measured in that study were within the range reported herein (~ 495 pmol/COC/hr glucose consumption and ~1, 145 pmol/COC/hr lactate production vs. 300 to 1965 pmol glucose/COC/hour and 732 to 2909.5 pmol lactate/COC/hour in the current study). As most studies in other species have examined metabolism in denuded oocytes, it is inappropriate to compare these data with those obtained here. Furthermore, the method of collection and number of cumulus cells differ, making comparison of COC data in other species difficult unless also corrected for DNA content.

The depletion of glucose and thus lack of glucose towards the end of maturation suggests that COC physiology might be compromised, however developmental data in the present study suggested otherwise. While numbers were small, one oocyte of nine that had depleted all the available glucose during IVM was able to form a blastocyst after ICSI. This either suggests that the COC was able to use alternate substrates for ATP production such as reusing excreted lactate or pyruvate or alternatively had completed the necessary glucose-requiring processes before glucose availability was limiting. In support of this, glucose consumption decreased during 20-30 hours of IVM. Of course it is not possible to determine when the COC effectively ran out of glucose during IVM, therefore either explanation is possible. The finding that COCs that had depleted all available glucose also had a reduced glycolytic index (60% vs. 95%) however, suggests a sensing mechanism enabling glucose to be diverted to pathways other than glycolysis, with ATP demand satisfied from other substrates. Additionally, the decrease in net pyruvate production also suggests that the COC and/or oocyte was able to either consume some of the

secreted pyruvate after glucose was depleted or secreted less in favour of conserving glucose. Increased β -oxidation of intracellular fatty acids as a source of ATP can also not be ruled out in these COCs that had depleted available glucose.

Maturation status did not impact either glucose or lactate (GL) metabolism. This suggests that the oocyte had a negligible effect on the overall COC metabolism as suggested by Sutton et al. (2003), who did not find the oocyte to influence COC metabolism. This will be explored in Chapter 4. Additionally, cumulus morphology did not influence COC metabolism. Given the physiological differences in follicle of origin between Cp and Ex COCs this result was unexpected. While compact (Cp) COCs had an increased GL metabolism when evaluated by individual complex, as also reported by Gonzalez-Fernandez et al., (2018), this difference was no longer present when values were normalised for DNA content, as the Cp cumuli contained many more cumulus cell than did expanded (Ex) COCs. This was perhaps a limitation of the equine study by Gonzalez-Fernandez et al., (2018) who reported that Cp COCs consumed significantly more glucose as results were not normalised for DNA content, which may have diminished the reported difference between Cp and Ex metabolism.

There were no differences in COC glucose or lactate metabolism during IVM between those oocytes that subsequently cleaved after ICSI and those that did not cleave; or between those that reached the blastocyst stage and those that did not. The association between COC metabolism during IVM and oocyte developmental competence has been evaluated in the cat, mouse and cow, and in all three species, increased glucose consumption during IVM was associated with increased developmental competence (Spindler et al., 2000, Preis et al., 2005 and Krisher and Bavister, 1999). The lack of association in this study could be due to species differences, the increased number of cumulus cells dampening the effect, or most likely the small number of blastocysts available for comparison. Further study is required to discover if COC glucose metabolism during IVM has predictive value in determining blastocyst development for the horse.

Temporal differences were observed in metabolism over the course of IVM, in that COCs consumed glucose and produced lactate at a lower rate during the final 10 hours of IVM. The glycolytic index remained constant however (119%; lactate : glucose ratio 2.37) suggesting that the change at the end of maturation represented an overall decrease in metabolism rather than a change of pathway. This is in contrast to studies in the mouse and the cow, which have found an increase in glucose consumption rates at the end of maturation but no corresponding increase in lactate production (Preis et al., 2005; Sutton et al., 2003). This increased glucose consumption suggests that additional pathways are utilised towards the end of maturation in these species other than glycolysis alone. The constant rate of glycolysis found in the present study supports the findings of Cetica et al. (2002) who reported that the activity of phosphofructokinase (PFK; a regulatory enzyme of glycolysis) did not change over the course of bovine IVM. In contrast, Harris et al., (2007) observed an increase in both glucose and lactate production in MII murine COCs compared to those at the GV stage, representing an overall increase in metabolism towards the end of maturation. Again, it is difficult to compare studies reliably, even in the same species, due to differences in culture and analytical methods. For example, the data from the Harris, 2007 study was derived from *in vivo* matured COCs or those originating from *in vitro* cultured follicles.

As considered in Section 1.3.2, a key component of meiosis is germinal vesicle breakdown (GVBD) which is known to be associated with an increase in glucose consumption via both glycolysis and the Pentose Phosphate Pathway (PPP) (Downs and Utecht, 1999, Herrick et al., 2006). There is wide species variation in the time of GVBD after onset of IVM; this occurs after 2 hours of a 12-14-hour IVM in the mouse, after 6-9 hours of a 24-hour IVM in the cow and after 16-20 hours of a 44-hour IVM in the pig (Donahue, 1968; Motlik and Fulka, 1976; Sirard and First, 1988). In a study examining maturation kinetics in the horse, GVBD occurred at approximately 12 hours after onset of IVM and progression to MII after 16 hours (Alm and Hinrichs, 1996), both events falling within the 10-20 hour culture window. Additionally, cumulus expansion occurs in the later part of IVM, at least in the mouse (Chen et al., 1993). Cumulus expansion increases glucose consumption

through the hexosamine biosynthesis pathway (HBP). It would be therefore expected that glucose consumption would be highest when both meiotic maturation and cumulus expansion are occurring. In the present study there was no significant increase in glucose metabolism during the time period that these processes would potentially be occurring (10-20 hours), however numbers of COCs were low. This will be explored further in Chapter 4.

This is the first study to examine oxygen consumption and its components in equine COCs and only the second to examine OCR in COCs of any species. The method used, proved to generate reliable and consistent data and has great potential to provide vital information on mitochondrial function during IVM and the subsequent stages of embryo development. Examining basal OCR revealed a tendency for it to increase over time (the opposite pattern to glycolysis) however the change was not statistically significant (42.48 ± 10.4 at the beginning of IVM vs. 60.58 ± 14.08 pmol/ng DNA/hour at the end of IVM). The trend is in agreement with both Johnson et al., (2007), who demonstrated an increase in pyruvate oxidation following GVBD in murine oocytes, and Sutton et al. (2003) who demonstrated increase in OCR over time in bovine COCs. Interestingly, basal OCR in the equine COCs in the present study was ~20 times higher than that reported for bovine COCS (per ng DNA; Sutton et al., 2003). This represents a significant species difference and highlights that unlike at least the bovine COC, the equine COC is not “mainly glycolytic”. It is interesting to note that this data is in agreement with that for denuded oocytes. A recent report of OCR in single metaphase II denuded equine oocytes using a novel Clark-type electrode reported an OCR of 10% of that measure in entire COCs in the present study; approx. 180 pmol/oocyte/hour (Obeidat et al., 2018). In contrast, reported OCR for denuded bovine oocytes was approx. 1.4 pmol/oocyte/hour (Sugimura et al., 2012) and 20-30 pmol/oocyte/hour for denuded porcine oocytes (Sturmey and Leese, 2003). The reason for the species differences could potentially be attributed to differences in ATP requirements or more likely, differences in triglyceride content and the proportion of which is subsequently oxidised. While intracellular lipid content has not been measured in equine oocytes, they are also very dark in colour, as in the pig, and have numerous

lipid-like vesicles present on electron microscopic evaluation (Grondahl et al., 1995) thus it is assumed that they have a high lipid content.

As was demonstrated for glucose and lactate metabolism, there were no differences in OCR attributable to cumulus classification. However, numbers of COCs in each cumulus category were small and this comparison would need to be repeated before any conclusion can be drawn. To the best of my knowledge OCR and GL metabolism have not been reported in the same oocytes or COCs; in the present study, these measurements were made possible with the *in situ* assessments of the Seahorse XFp analyser. Interestingly, while glucose consumption was within the range found from the COCs in the previous microdrop experiments, lactate production was much lower and hence so was the glycolytic index. Unfortunately, this was not explored further but could be due to differences in volume (60 μ l/COC vs. 10 μ l) or group vs. single culture. An additional difference is the fact that the media was mixed and reoxygenated several times during the Seahorse experiments compared to the static nature of the microdrop culture. Reoxygenation could have increased OXPHOS and therefore proportionately decreased glycolysis to explain the lowered lactate production. Overall, as the ratio of lactate : glucose was not correlated to OCR but the GL metabolism was, it suggests an absence of a reciprocal relationship but rather that COCs either had a “quiet” or “noisy” level of metabolism overall.

Mitochondrial function testing, by examining the efficiency of OXPHOS, has been reported widely for somatic cells, but there is only one report for bovine oocytes (Sugimura et al., 2012) and one in equine oocytes (Obeidat et al., 2018). The equine study by Obeidat et al., used oligomycin in isolation and therefore only coupled OCR could be determined. The findings reported here for equine COCs are very similar to those reported previously for bovine denuded oocytes by Sugimura et al., (2012). While somatic cells have a non-mitochondrial OCR of approximately 10% of basal (basal OCR = 100%) (Wu et al., 2007), equine COCs in this study and bovine oocytes in the Sugimura study had a non-mitochondrial OCR of 38% and 35% respectively. Non- mitochondrial OCR can be attributed to the production of ROS or to the action

of oxygen-consuming enzymes such as NADPH oxidase, xanthine oxidase and squalene mono-oxygenase, as demonstrated in rabbit embryos (Manes and Lai, 1995). The efficiency with which OCR in equine COCs was coupled to ATP production via ATP synthase varied between 47 – 58% and did not change over the time course of IVM. This is in contrast to the bovine denuded oocyte in which coupling efficiency decreased from 40.9% in immature oocytes to 25% in Metaphase II oocytes (Sugimura et al., 2012). The fall in coupling efficiency was attributed to an increase in proton leak in the bovine oocytes, which did not occur in the equine COCs (17% throughout IVM). Interestingly, the previous equine study by Obeidat et al., reported an 85% coupling efficiency. However, it is difficult to compare this result, and those from the Sugimura study, given that both were performed on individual denuded MII oocytes rather than COCs in group culture. A drawback of this present study is that COCs were not cultured individually due to lack of sensitivity of the Seahorse XFp analyser and hence meiotic status was unknown at the end of the maturation period.

Surprisingly, there was an 80% reduction in the spare respiratory capacity of equine COCs at the end of IVM (IVM + 28 hours) compared to initially (IVM + 4 hours), however the increase in OCR by the end of maturation (< 20 pmol/ng DNA/hour) cannot account for all of this loss of spare capacity. A decrease in spare capacity is a marker of mitochondrial dysfunction (Brand and Nicholls, 2011) and potentially has detrimental consequences for oocyte developmental competence, especially given the requirement for a high electrical potential across the mitochondrial membrane to sustain calcium oscillations at fertilisation (Dumollard et al., 2004). In the murine study by Sugimura et al., a decreased spare capacity was found only in aged oocytes following IVM. This is interesting, given that recent reports in the equine literature suggesting that overnight holding of COCs prior to initiating IVM affects oocyte maturation kinetics and may result in a significant portion (up to 42.3%) of oocytes becoming mature after only 20-22 hours in maturation culture (Dini et al., 2016; Rodríguez et al., 2016). This could potentially cause some oocytes to become “aged” by 28 hours of IVM. However, the study by Dini et al., performed with oocytes recovered from slaughterhouse tissue, contrasts with the findings of Choi

et al. (2015) in which oocytes from live mares were held overnight and only 28% were at MII after 20 hours maturation culture, vs. 60% at 24 hours. Moreover, Jacobson et al. (2010) reported that blastocyst rates did not differ between maturation times of 24 vs. 30 hours for oocytes recovered by TVA and held overnight before onset of maturation (33% blastocysts in both groups), and that *in vivo* data suggest oocytes remain viable for at least 6-12 hours after ovulation (Ginther, 1992; Woods et al., 1990). Overall, these findings indicate that oocyte aging is unlikely to explain the reduction in spare mitochondrial capacity. Another possibility is that the cumulus cells, nearing the end of their physiological role have no further requirement to maintain 'metabolic scope' and as an evolutionary adaptation this lack of mitochondrial spare capacity signals their demise (Peterson et al., 1990)

Conclusions

The work presented in this chapter has for the first time described the pattern of glucose, lactate and oxidative metabolism in equine COCS during the 30 hours of IVM using CoRe methods and the Seahorse XFp analyser. The findings demonstrate that like other species, equine COCs utilise the majority of glucose via glycolysis however equine COCs have a unique temporal pattern of glucose metabolism. It is also shown that unlike at least the bovine COC, the equine COC is not "mainly glycolytic" as OXPHOS also plays a major role in energy metabolism suggesting oxidation of an additional substrate(s). Additionally, as analysis was performed using clinical IVM conditions, clinically relevant findings are described: shortcomings in glucose availability during IVM culture were identified, and marked decreases in mitochondrial efficiency were uncovered at the completion of IVM. These findings will form the basis of further studies in subsequent chapters to optimise equine IVM in terms of glucose availability and to explore the addition of substrates that would potentially prevent such a dramatic loss of spare mitochondrial capacity by the conclusion of maturation.

Chapter 4: The effect of oxygen and pyruvate concentration on carbohydrate metabolism and oxygen consumption in equine cumulus oocyte complexes.

4.1 Introduction

Equine oocyte *IVM* conditions have not been critically evaluated. Indeed, in all species studied thus far, *in vitro* matured oocytes are inferior to their *in vivo* matured counterparts and perform sub-optimally in IVP systems. The benefits of low oxygen concentration during embryo culture are widely accepted and embryo culture at 5% oxygen is nearly universal for animals and becoming so for human embryo culture (Wale and Gardner, 2016). In contrast, the ideal oxygen tension for IVM is still under debate, with conflicting results from different studies. This is despite the fact that the oxygen concentration in the ovarian follicle is between 1 and 5.5% in the human (VanBerkom et al., 1997). Additionally, as the antrum grows during follicular growth and then the cumulus cells expand in the large pre-ovulatory follicle, the oxygen-consuming cell mass increases, and the oocyte becomes farther away from follicular vascular supply and thus also oxygen (Tamanini and De Ambrogi, 2004; Thompson et al., 2015). This lack of apposition could potentially be even more important in the equine pre-ovulatory follicle, which can reach upwards of 45 mm in diameter before ovulation. Interestingly, it has been demonstrated through mathematical modelling that little oxygen is removed by murine and bovine cumulus cells (< 0.5%) as they are mainly glycolytic (Clark et al., 2006) as such, the oxygen gradient between the follicle wall and the oocyte will be small. In contrast, results from Chapter 3 for the equine COC suggest this gradient may be larger if the high OCR was maintained *in vivo*.

An early study by Gwatkin and Haidri, (1974) demonstrated a clear benefit of 5% oxygen during IVM on the meiotic competence of hamster oocytes. However, reported benefits of low oxygen are not unanimous across species. In the pig, atmospheric oxygen (20%) during IVM has been shown to be superior in terms of blastocyst development in some studies (Kang et al., 2011; Park et al., 2005) yet in other studies, inferior in terms of both blastocyst development and quality

(increased cell number and decreased DNA fragmentation with IVM at 5% vs. 20%; Iwamoto et al., 2005; Karja et al., 2004). In cattle, one study reported increased blastocyst numbers after IVM at 20% vs. 5% oxygen (Oyamada and Fukui, 2004), however others found a positive effect of low oxygen (5%) during IVM on blastocyst numbers (Bermejo-Álvarez et al., 2010; Hashimoto et al., 2000; Kumar et al., 2015), and also on the expression of genes related to oocyte developmental competence (Bermejo-Álvarez et al., 2010). In the mouse, Banwell et al., (2007) and Preis et al., (2007) reported that while there were no differences in developmental rates or live birth rates between oocytes matured in low and high oxygen concentration, total cell and trophectoderm cell numbers were increased when oocytes underwent IVM at 5%. In the only study to examine the effect of oxygen concentration during IVM on post implantation development, murine foetal and placental weights were decreased with IVM at 5% vs. 20% (Banwell et al., 2007). There are no studies directly comparing outcomes between different oxygen concentrations for IVM in the human, however clinical success has been reported at both 5% (Child et al., 2001; Junk et al., 2012; Walls et al., 2015) and 20% oxygen (Filali et al., 2008). Clearly, the optimal concentration of oxygen during IVM requires further investigation.

If the approach of mimicking the *in vivo* environment is adopted, an important substrate not included in M199 media but known to be present in follicular fluid is pyruvate (Gerard et al., 2000). Pyruvate is important in order to meet the ATP requirements of the oocyte (Gardner and Leese, 1990; Harris et al., 2005; Leese and Barton, 1984) and for ROS scavenging (O'Donnel and Tormey et al., 1987). As detailed in Section 1.5.4 the cumulus cells metabolise glucose via glycolysis for both their own ATP production and for providing pyruvate to the oocyte (Harris et al., 2009) and the oocyte subsequently utilises a portion of this pyruvate for ATP production through the TCA cycle and OXPHOS (Steeves and Gardner, 1999). It has also been shown that cumulus cells will prioritise pyruvate secretion over meeting their own metabolic ATP requirements in order to maintain redox balance (O'Donnel and Tormey et al., 1987). Given the net pyruvate production in COCs reported in Chapter 3, it is possible that by adding pyruvate to the medium before

IVM commences, glucose may be spared and used for other functions. Furthermore, it has been shown that the combined presence of glucose and pyruvate results in the highest maturation rates for mouse COCs (Downs and Hudson, 2000). While addition of pyruvate at varying concentrations (0.1 to 1 mM) during equine IVM has been reported by some (Foss et al., 2013; Galli et al., 2002), the effect on metabolic, meiotic or developmental parameters has not been studied. Follicular-fluid pyruvate concentrations in the mouse, human, cow and mare are 0.38, 0.26, 0.01-0.05 and 0.03-0.13 mM respectively (Gerard et al., 2000; Harris et al., 2005; Leese and Lenton, 1990; Orsi et al., 2005). Interestingly, in the human this is 3 to 5 times the plasma concentration (Leese et al., 1986) emphasising its importance to the developing oocyte.

It is also of interest to consider what elements are responsible for the temporal differences in glucose consumption reported in Chapter 3. It could be postulated that ATP-requiring meiotic events such as GVBD within the oocyte would drive metabolism in the COC as a whole, which has been demonstrated in the mouse and pig (Matsuno et al., 2016; Sugiura et al., 2005), however in the cow this was not the case as the presence of the oocyte did not alter glucose metabolism compared to cumulus cells only (oocyte-ectomised COCs) (Sutton et al., 2003). Follicle stimulating hormone (FSH) has also been shown to affect glucose metabolism in the COC, possibly linked to its role in stimulating cumulus expansion. As cumulus expansion requires hyaluronic acid, an end product of the hexosamine biosynthesis pathway (HBP), FSH addition increases glucose demands through the HBP. This has been shown in cattle where glucosamine addition, an alternative substrate for the HBP, decreased glucose consumption (Sutton-McDowall et al., 2005).

Summary

The data in Chapter 3 indicated that equine COCs consume glucose avidly and export pyruvate into the medium. In addition, the spare respiratory capacity of the COC mitochondria was significantly decreased at the end of maturation. Taken together, these findings suggest that the current IVM conditions for equine COCs are suboptimal. Data is ambiguous on environmental aspects such as oxygen concentration and medium composition that could address these inadequacies therefore further studies are required. As such, the effect of lowering the oxygen concentration and adding pyruvate at physiological concentrations on COC metabolism and meiotic potential was investigated. Further, from a basic biology viewpoint, the temporal pattern of glucose, lactate and pyruvate metabolism was scrutinized.

4.2 Objectives

1. Evaluate the effect of addition of 0.15 mM pyruvate to IVM medium, and the effect of decreased oxygen concentration (5% vs. 21%), on COC maturation rate and glucose, pyruvate and lactate (GPL) metabolism (Experiment 4.1).
2. Evaluate effect of IVM medium pyruvate concentration (0 mM, 0.15 mM, 0.5 mM) on basal OCR and mitochondrial function at the completion of IVM (Experiment 4.2).
3. Evaluate the temporal changes in GPL metabolism over the 30-hour duration of IVM (0-10 hours, 10-20 hours, 20-30 hours) in the presence of pyruvate (Experiment 4.3, Study 1). Using this model, evaluate:
 - a. The impact of oocyte presence within a COC on GPL metabolism (intact COC vs. granulosa only) (Experiment 4.3, Study 2)
 - b. The impact of presence of FSH (0 vs. 5 mU/ml) in the maturation media on GPL metabolism. (Experiment 4.3, Study 3)

4.3 Materials and methods

General methods

COCs were recovered from abattoir-derived ovaries, classified as compact (Cp), expanded (Ex) or corona radiata-only as previously described in Section 2.1. Granulosa was trimmed using a 21-G needle (visual trimming [VT]) or a 290- μ m pipette (pipette trimming [PT]) in an attempt to minimise variation in cumulus mass. See results of each experiment for details. COCs were then held overnight as previously described in Section 2.1.

Experiment 4.1

When COCs were removed from the holding vial Cp and Ex COCs were divided equally and without any further selection between conditions, so that for each collection day, each maturation condition contained equal numbers of each cumulus type. Maturation in all groups was carried out in individual 15- μ l droplets (increased from 10- μ l in an attempt to prevent glucose depletion; Section 3.4.1.). There were 12 droplets in each dish; COCs were placed in nine of them and three remained empty and were used as reference droplets. Dishes were continuously incubated for the entire 30-hour IVM period in one of four IVM conditions as follows (as indicated in Figure 4.1): **Group 21C:** Control Maturation Medium (no pyruvate; M199 with Earle's salts, 10% FBS, 25 μ g/ml gentamicin with 5 mU/ml FSH [Sioux Biochemical Inc., Sioux Center, IA]) and maturation at 5% CO₂ in air (21% O₂). **Group 5C:** Control Maturation Medium (as above, no pyruvate) and maturation at 5% CO₂, 5% O₂ and 90% N₂. **Group 21P:** Maturation Medium with added pyruvate (control medium as above with the addition of 0.15 mM Pyruvate) and incubation at 5% CO₂ in air. **Group 5P:** Maturation Medium with added pyruvate and incubation at 5% CO₂, 5% O₂ and 90% N₂. After the maturation period was completed, COCs were denuded and classified as Metaphase II (MII), intact or degenerated (DEG) as described in Section 2.1.4. All denuded cumulus cells from each COC were placed in a 96-well plate and kept at -20° C for later DNA quantification analysis. Spent media samples were frozen at -80° C and analyses of thawed samples carried out as described in Section 2.2

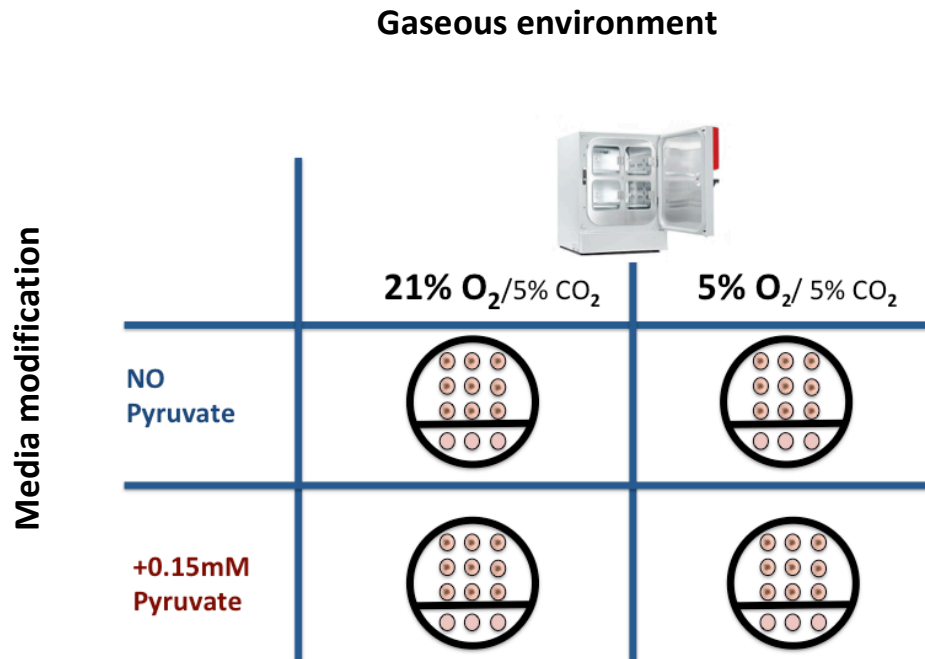


Figure 4.1: Schematic diagram of experimental design for Experiment 4.1.

Experiment 4.2

In vitro maturation was carried out directly in a Seahorse plate (180 µl medium per well) in groups of 3 Cp COCs (see Section 2.4.3 for full description) in one of the following three conditions; 1) Control Maturation Medium (M199 with Earle's salts, 10% FBS, 25 µg/ml gentamicin with 5 mU/ml FSH and maturation at 5% CO₂ in air (21% O₂). As for Experiment 4.1, Control Maturation Medium [**Group 21C- 0mM**]; 2) Control Maturation Medium as above with the addition of 0.15 mM pyruvate and incubation at 5% CO₂ in air [**0.15mM**]; and 3) Control Maturation Medium as above with the addition of 0.5 mM pyruvate and incubation at 5% CO₂ in air [**0.5mM**]. The Seahorse plate was removed from the incubator after 28 hours of IVM and placed in the Seahorse XFp analyser. After calibration, three basal oxygen consumption rate readings (pmolO₂/well/min), were recorded before a series of respiratory inhibitors were added sequentially to each well to reach the following working concentrations; 1) Oligomycin [1 µM] 2) FCCP [5 µM] 3) Antimycin/Rotenone [2.5 µM] (See Section 2.4.3 for full description of method.) After the readings were obtained, COCs were placed into a plate and frozen for DNA quantification.

Experiment 4.3

Only Cp COCs were used and all were individually cultured. A 10- μ l droplet was prepared for each individual COC in each of three separate dishes, and each COC was moved every ten hours from one dish to another (See Section 2.2.4 for full description). IVM was carried out at 5% CO₂ in air, with other conditions differing as follows; In **Study 1**, maturation was carried out in pyruvate-containing maturation medium as per Group 21P in Experiment 1. In **Study 2**, maturation medium and conditions were performed, as for Study 1, but in each replicate, half of the droplets contained granulosa cells only that were visually estimated to be the same size as the COC. Mural granulosa cell samples were obtained from dishes in which a Cp COC was located (i.e. the same follicle). In **Study 3**, COCs were divided between two treatments: Maturation medium with pyruvate, incubated in 5% CO₂ in air, as per Group 21P (+ FSH) and identical maturation medium and conditions but without FSH (- FSH). The oocytes were denuded and classified, and cumulus cells from each COC and the granulosa cells cultured separately and spent media were analysed, as described for Experiment 4.1.

Statistical analysis

In Experiments 4.1 and 4.3, the primary outcome variables were glucose depletion, lactate and pyruvate accumulation rates (expressed as pmol/ngDNA/hour). Univariate analyses using the Chi-squared test and student's t-test were initially performed to explore possible associations between measured variables. Following univariate analyses, multivariable linear regression models (Experiment 4.1) and mixed multivariate linear regression models, with COC ID included as a random effect to account for repeated measures over time (Experiment 4.3) were fitted to verify associations while accounting for potentially confounding variables. All potential explanatory variables were offered to the initial model (medium, oxygen concentration, date of replicate (batch), trimming, maturation status and cumulus classification). Interaction terms were included if biologically plausible and retained if they improved model fit. Final variable selection was by a backward-stepwise method taking $p < 0.25$ as the criterion for retaining the variable in the final model. The likelihood ratio test was used for comparison of competing models.

In Experiment 4.2, the third OCR measurement was used to represent the basal OCR and the measurement representing maximal response was used for all inhibitor OCR data. Data were evaluated as pmol O₂/COC/hour and pmol O₂/ngDNA/hour. One-way ANOVA was used to explore associations between groups.

For full details on statistical analyses including criteria for data cleaning and replicates see Section 2.5.

4.4 Results

4.4.1 Experiment 4.1- Effect of pyruvate addition and oxygen concentration during IVM on GPL metabolism

One hundred and thirty two COCs were evaluated over four replicates (Table 4.1). Three were lost due to technical errors and a further seven during data cleaning. Data for a total of 122 COCs were available for statistical analysis.

	21% OXYGEN		5% OXYGEN			
REPLICATE	+P	-P	+P	-P	TRIM	DNA
	Group 21P	Group 21C	Group 5P	Group 5C		(mean; ng)
1	10(9)	10(9)	10 (9)	10 (6)	VT	139 ^a
2	8 (8)	8 (8)	8 (8)	8 (8)	VT	136 ^a
3	10 (10)	10(9)			PT	43 ^b
4	10 (10)	10(9)	10 (10)	10(9)	PT	64 ^b

Table 4.1: Allocation of COCs to maturation conditions for Experiment 4.1. Abbreviation +P denotes addition of 0.15 mM Pyruvate, -P denotes control media. TRIM indicates method used for granulosa trimming; VT = visual trimming with 21-G needle and PT indicates pipette trimming (290- μ m). Numbers in parenthesis represent final number of COCs analysed. Different superscripts indicate significant differences within columns ($p < 0.05$).

Cumulus oocyte complexes with compact cumulus (Cp) accounted for 54.9% of COCs analysed and expanded COCs (Ex), 45.1 %. Metaphase II rate was higher in Ex (60%) than Cp (22.4%; $p = 0.01$). Maturation rate did not vary over the four conditions (35-43.5%; $p > 0.05$).

Glucose consumption and lactate production were positively correlated to DNA content of the COC in all four IVM conditions ($r^2 = 0.3$; $p < 0.001$). However, there was no association between pyruvate production and COC DNA content (Figure 4.2). In the control standard conditions (Group 21C), 2.9% (1/35) of COCs depleted all available glucose from the 15- μ l drop compared to 16.2%-26.1% in the other three conditions.

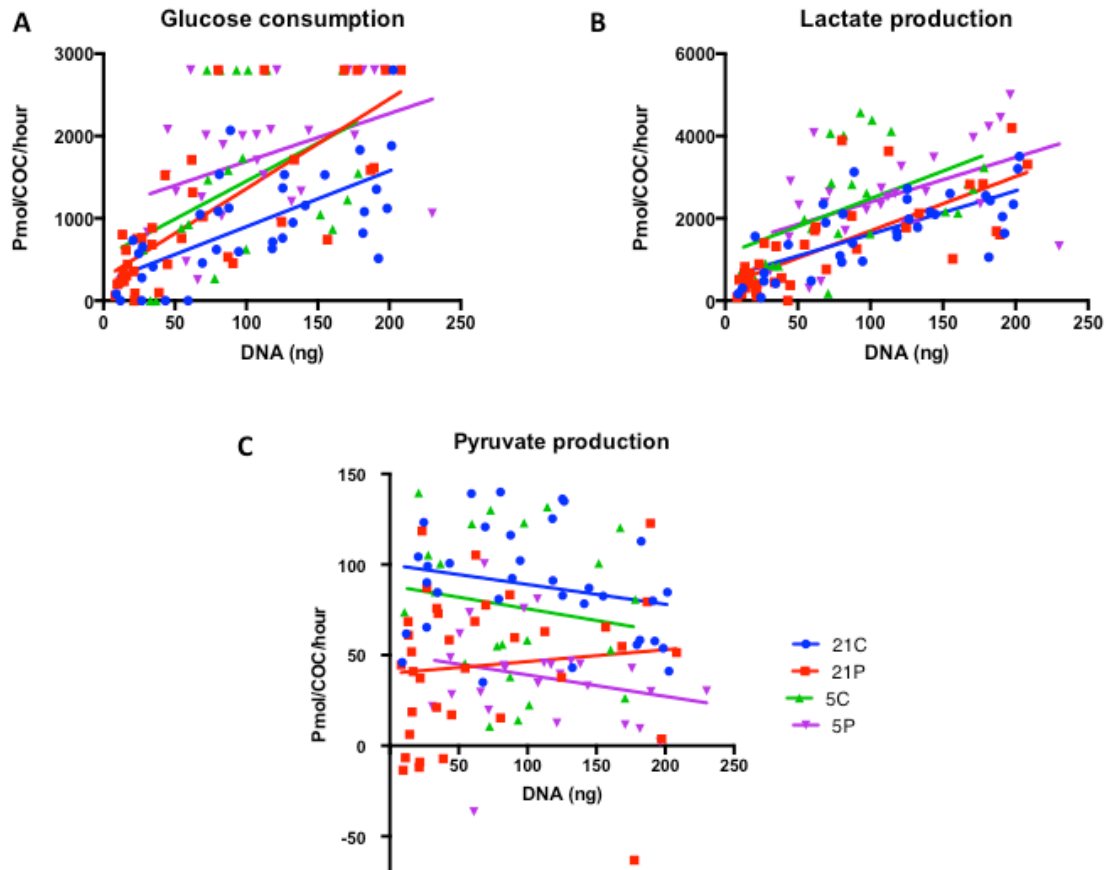


Figure 4.2: Glucose consumption (A) lactate (B) and pyruvate production (C) rates regressed on COC DNA content for the four IVM culture conditions as follows; 21C (blue) = Control maturation medium and maturation at 5% CO₂ in air (21% oxygen); 21P (red) = Pyruvate containing Maturation Medium (control medium as above with the addition of 0.15 mM Pyruvate) and incubation at 5% CO₂ in air (21% oxygen); 5C (green) = Control maturation medium and maturation at 5% CO₂, 5% O₂ and 90% N₂; 5P (purple) = Pyruvate containing maturation medium and incubation at 5% CO₂, 5% O₂ and 90% N₂. Associations between glucose consumption rate and DNA content, and between lactate production rate and DNA content were statistically significant ($p < 0.01$). Pyruvate production rates were independent of DNA content.

In COCs for which glucose was not limiting (COCs that did not deplete all available glucose during IVM; $n = 103$), the effect of oxygen concentration and addition of pyruvate to the media on glucose consumption, lactate production and pyruvate production rates could be evaluated. The addition of 0.15 mM pyruvate increased glucose consumption at both 5% and 21% oxygen (21P and 5P) but there was no corresponding increase in lactate production (Figure 4.3). At 5% oxygen, the addition of pyruvate (Group 5P) decreased the lactate : glucose ratio and thus decreased the glycolytic index (70 vs. 100%; $p = 0.001$). This condition (Group 5P) also resulted in significantly decreased pyruvate production rates compared with the other three conditions (0.6 vs. 1.6-2.1 pmol/ng DNA/hour; $p = 0.02$). These univariate findings were confirmed following multivariable linear regression modelling (See Table A3.1, Appendix 3). Degree of trimming was retained in the final model.

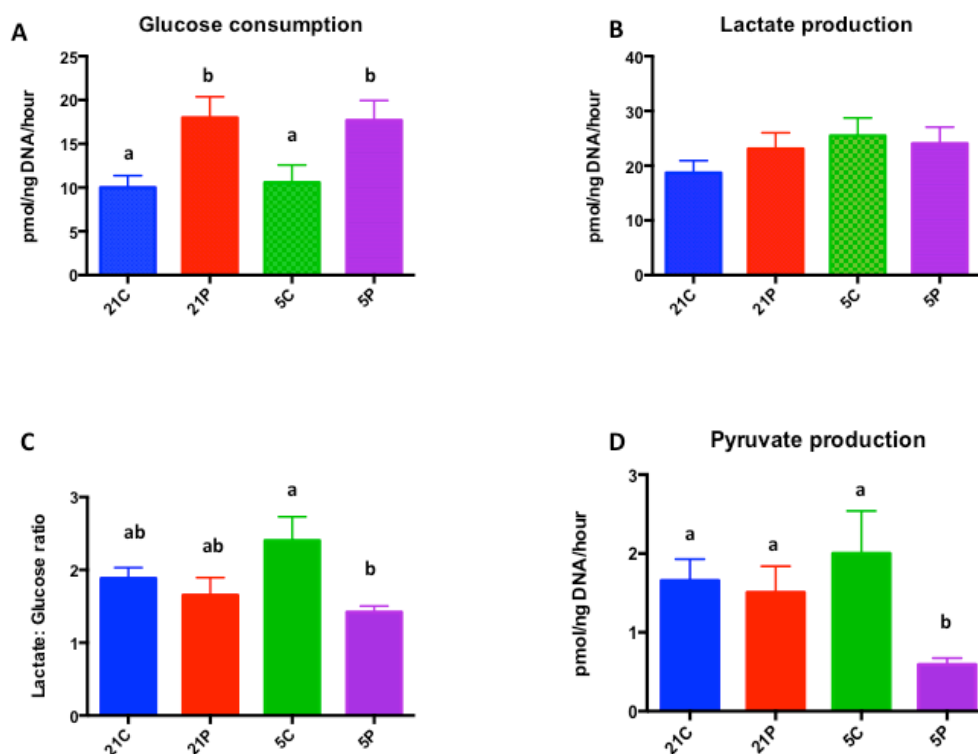


Figure 4.3: The effect of 0.15 mM pyruvate and oxygen concentration (5% vs. 21%) on glucose consumption (A), lactate production (B), lactate : glucose ratio (C) and pyruvate production (D) during IVM. Condition abbreviations on X axes are as follows; 21C (blue) = Control maturation medium and maturation at 5% CO₂ in air (21% oxygen); 21P (red) = Pyruvate containing Maturation Medium (control medium as above with the addition of 0.15 mM Pyruvate) and incubation at 5% Co₂ in air (21% oxygen); 5C (green) = Control maturation medium and maturation at 5% CO₂, 5% O₂ and 90% N₂; 5P (purple) = Pyruvate containing maturation medium and incubation at 5% CO₂, 5% O₂ and 90% N₂. Bars represent mean \pm SEM and different superscripts denote significance differences between groups ($P < 0.05$).

4.4.2 Experiment 4.2: Effect of pyruvate concentration on OCR and mitochondrial function

Forty-five compact COCs (15 in each condition) were used for this experiment over three replicates. Data from six COCs in the 0.15 mM Pyruvate group were excluded from subsequent analysis due to biologically implausible OCR readings.

There was a tendency for the COCs in the 0.5 mM Pyruvate group to have a higher basal OCR however this was not significant ($p = 0.2$; Figure 4.4). Pyruvate concentration (0 - 0.5 mM) had no significant effect on any measure of mitochondrial function (Figure 4.5).

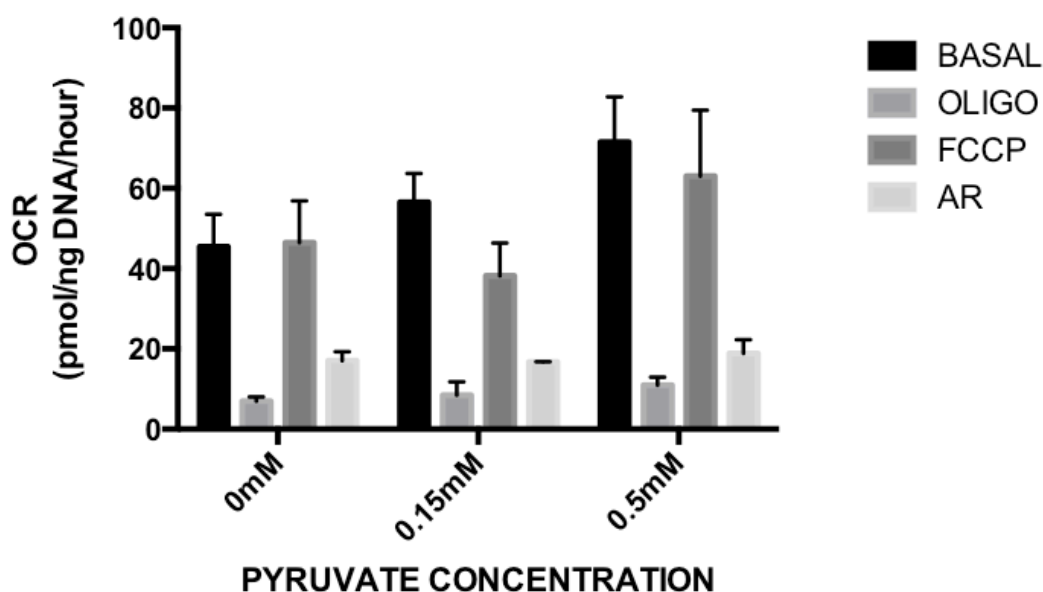


Figure 4.4: Effect of pyruvate concentration during IVM on oxygen consumption rate (OCR) using respiratory inhibitors to evaluate mitochondrial function ($n = 39$). Each bar represents the mean OCR after addition of the inhibitor stated (\pm SEM). Abbreviations as follows: OLIGO: Oligomycin; AR: Antimycin/rotenone.

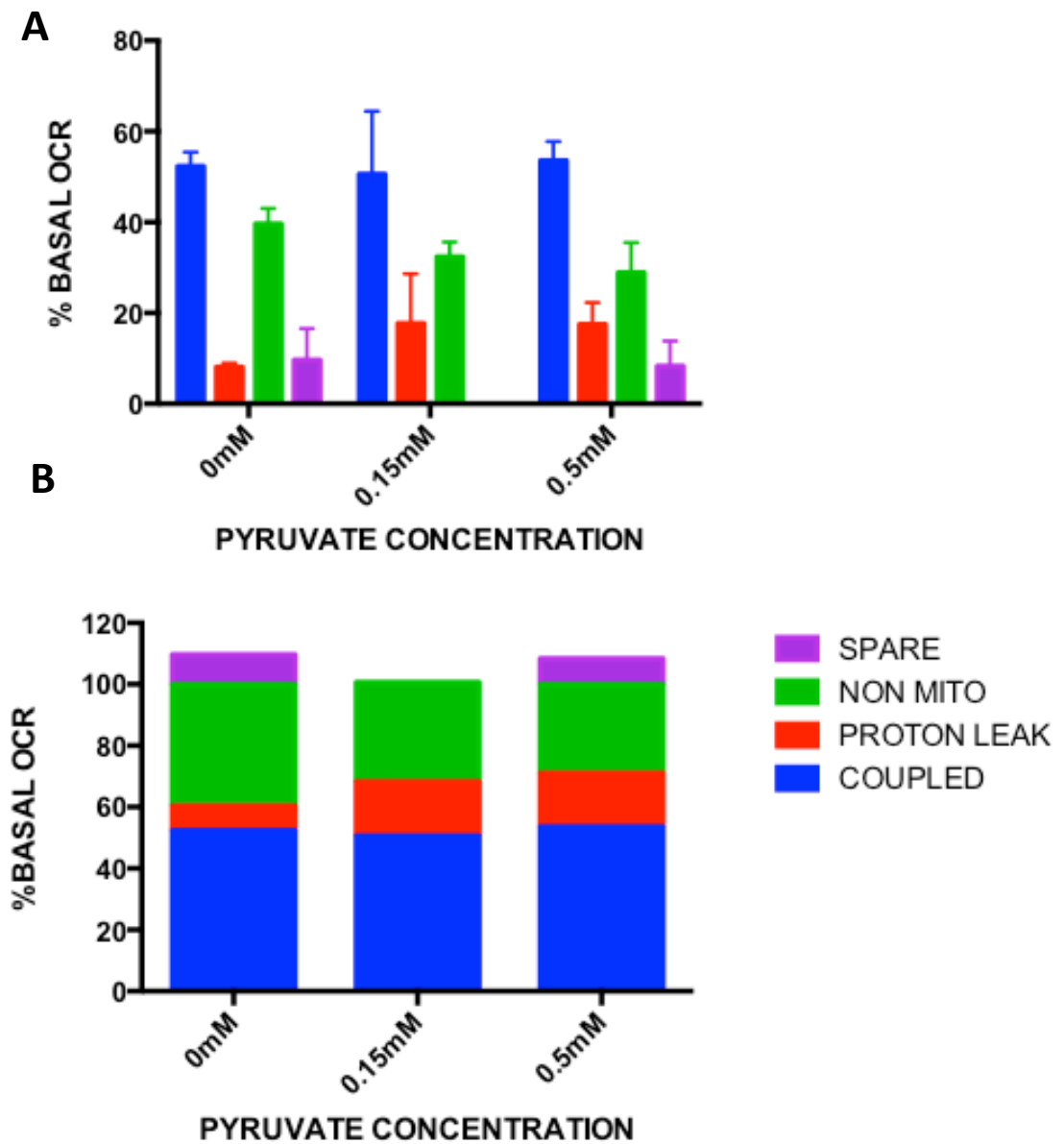


Figure 4.5: Effect of pyruvate concentration during IVM on mitochondrial function as determined following the sequential addition of respiratory inhibitors. Oxygen consumption rate (OCR) coupled to ATP production (BLUE), OCR associated with proton leak (RED), non-mitochondrial OCR (GREEN) and spare respiratory capacity (PURPLE) are expressed as % basal OCR. Mean \pm SEM is presented in Panel A and mean presented in Panel B.

4.4.3 Experiment 4.3- Temporal changes in GPL metabolism

One hundred and twenty-four COCs were evaluated over six replicates. One COC was lost due to technical issues and 15 were excluded from further analysis during data cleaning. A total of 108 COCs remained for statistical analyses.

REPLICATE	COC+FSH	COC NO FSH	GRAN ONLY (+FSH)	TRIM	DNA (mean; ng)
1	10 (5)			COR	67 ^a
2	18 (15)	10 (9)	10 (7)	NONE/COR	107 ^b
3	10 (10)	10 (8)		VT	120 ^b
4	10 (10)			PT	74 ^a
5	10 (9)	10 (10)	10 (10)	PT	83 ^a
6	8 (8)		8 (7)	PT	90 ^a

Table 4.2: Cumulus oocyte complex allocation for Experiment 4.3 (Studies 1-3). Abbreviations as follows; COC + FSH = cumulus oocyte complex in control Maturation Media containing FSH (5mU/ml). GRAN = Granulosa cells only. TRIM = method used for granulosa cell trimming; COR= corona only, no trimming (NONE), VT = visual trimming using 21G needle; PT = pipette trimming (290 µm). Numbers in parenthesis represent final number of COCs analysed. Different superscripts indicate significant differences within columns ($p < 0.05$).

Study 1: Temporal changes in GPL metabolism over the course of IVM in the presence of pyruvate.

Data for 57 COCs over six replicates were analysed for this study (COC + FSH; Table 4.2). Glucose consumption increased during 10-20 hours of culture, with a subsequent decrease during 20-30 hours (Figure 4.6). There was a simultaneous decrease in lactate production during 20-30 hours (Figure 4.6) resulting in the lactate : glucose ratio being unaffected by maturation period. Pyruvate production remained similar during 0-10 and 10-20 hours but significantly increased during the final period of IVM (20-30 hours; Figure 4.6). These findings remained significant in the mixed multivariate linear regression model when all potential confounding variables were taken into account (batch number remained in the final model, see Table A3.2, Appendix 3).

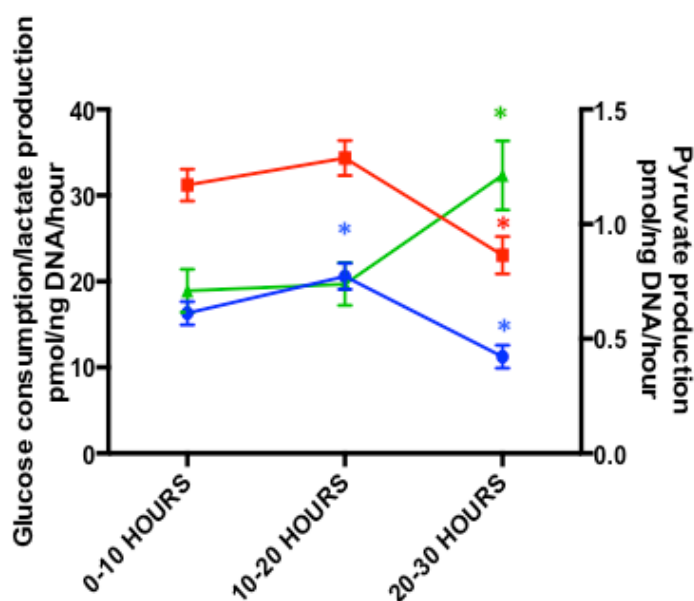


Figure 4.6: Effect of IVM period (0-10, 10-20, 20-30 hours) on glucose (blue) consumption, lactate (red) and pyruvate (green; right axis) production rates. Mean \pm SEM are depicted; blue asterisks denote significant differences between time periods in glucose consumption rate (10-20 and 20-30hours); red asterisks denote a significant decrease in lactate production rate (20-30hours) and green asterisks a significant increase in pyruvate production rate (20-30hours). (All $p < 0.05$)

Study 2: The impact of oocyte presence on GPL metabolism (intact COC vs. granulosa only).

Data from 56 COCs/granulosa samples over three replicates were analysed for this study (COC + FSH and GRAN only; Table 4.2). Glucose, lactate and pyruvate metabolism of COCs was significantly different to that of granulosa cells alone during at least one time period over the 30 hours of IVM (Figure 4.7; yellow line). As shown in Study 1, GPL metabolism varied over time for COCs, however in contrast, when granulosa cells were cultured separately in maturation media without an oocyte, glucose and lactate metabolism remained constant over time (Figure 4.7A and 4.7B). In addition, granulosa cells cultured alone had decreased pyruvate production during 20-30 hours, which was the opposite temporal pattern to that of intact COCs (significant increase at 20-30 hours; Figure 4.7D). These findings were confirmed in the mixed multivariate linear regression model (Table A3.3; Appendix 3). No other variables were found to affect any of the parameters significantly and were therefore not retained in the final model.

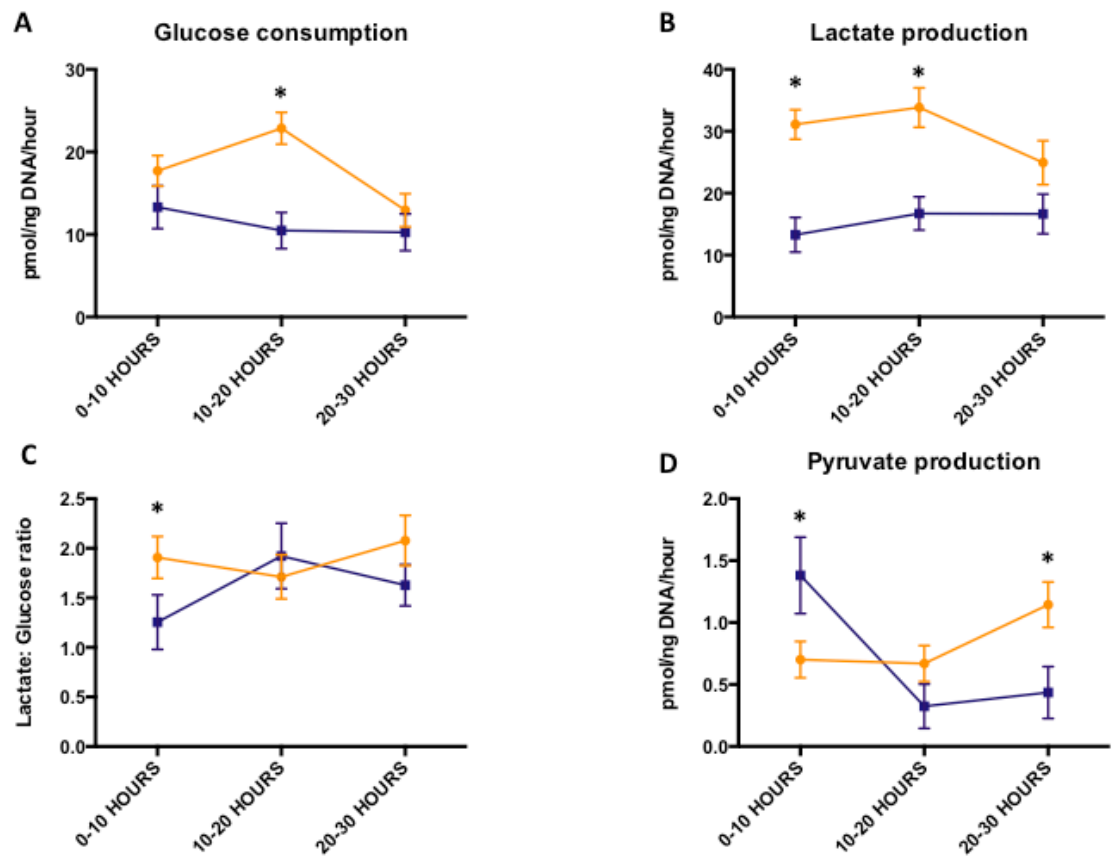


Figure 4.7: Effect of oocyte presence (COC; yellow) vs. granulosa cells only (blue) on glucose consumption (Panel A), lactate production (Panel B), lactate : glucose ratio (Panel C) and pyruvate production (Panel D) over the time course of IVM. Mean \pm SEM are depicted and asterisks represent significant difference between conditions at that time point ($p < 0.05$).

Study 3: Impact of the presence/absence of FSH on GPL metabolism.

Data from 61 COCs over three replicates were analysed for this study (COC + FSH and COC NO FSH; Table 4.2). The presence of FSH did not affect the temporal pattern of glucose consumption or lactate and pyruvate production, however there were significantly increased lactate and pyruvate production rates during both 10-20 and 20-30 hours of IVM in the presence of FSH (Figure 4.8). These findings were confirmed in the mixed multivariate linear regression model (Table A3.4; Appendix 3). No other variables were found to affect any of the parameters significantly and were therefore not retained in the final model.

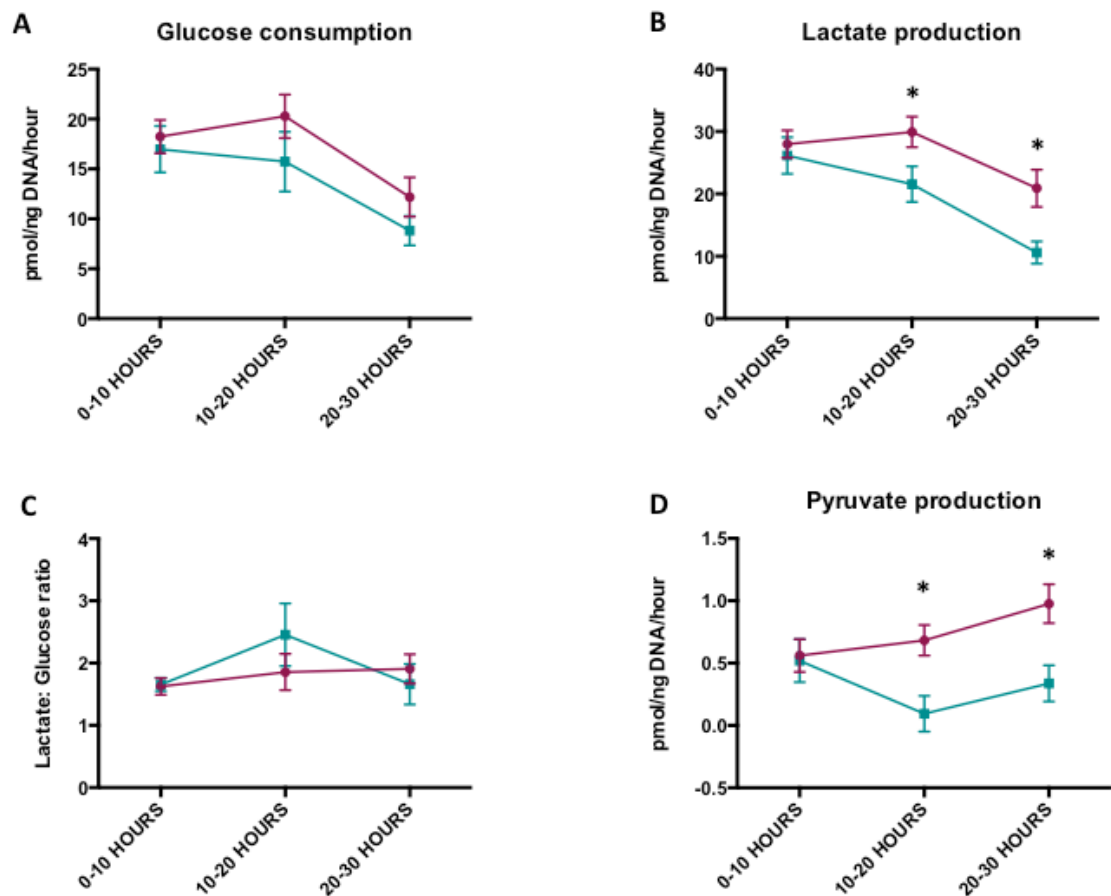


Figure 4.8: Effect of FSH (5mU/ml purple and 0mU/ml FSH teal) on glucose consumption (Panel A), lactate production (Panel B), lactate : glucose ratio (Panel C) and pyruvate production (Panel D) over the time course of IVM. Data are presented as mean \pm SEM, asterisks represent significant difference between conditions at that time point ($p < 0.05$).

4.5 Summary findings

- Provision of 0.15 mM pyruvate increased glucose consumption at both 5% and 21% oxygen.
- At 5% oxygen, the addition of pyruvate decreased the glycolytic index compared to 21% oxygen. This condition (Group 5P) also resulted in significantly decreased pyruvate production rates compared with the other three conditions.
- Pyruvate production was not correlated to COC DNA content.
- The proportion of COCs depleting all available glucose decreased to 2.9% in Group 21C as a result of technical interventions (trimming excess granulosa and increasing droplet size to 15 μ l).
- Pyruvate concentration had no significant effect on basal OCR or on any measure of mitochondrial function.
- GPL metabolism varied over time for COCs, however, glucose and lactate metabolism remained constant over time in granulosa cells that were cultured separately in maturation media without an oocyte.
- The presence of FSH did not affect the temporal pattern of glucose consumption or lactate and pyruvate production, however there were significantly increased lactate and pyruvate production rates during both 10-20 and 20-30 hours of IVM in the presence of FSH.

4.6 Discussion

In this study it was shown that provision of pyruvate and lowering the oxygen concentration during IVM resulted in measurable changes in the metabolism of equine COCs. Glucose consumption was increased in media containing 0.15 mM pyruvate, independent of oxygen concentration. At 5% oxygen (Group 5P), there was a reduction in pyruvate production. Multivariable analysis confirmed pyruvate concentration to be a significant explanatory variable. Given the known role of pyruvate production in antioxidant defence, it is interesting that there was a reduction in pyruvate production at 5% oxygen but only in media already containing pyruvate (Group 5P). Assuming that pyruvate secretion reflects the oocyte's response to oxidative stress, these data support the notion that these conditions result in the least oxidative stress for the COC and hence production is lowered and glucose is spared for other pathways. In further support of this proposition, only 70% of glucose consumed was converted to lactate in Group 5P vs. 100% in the other conditions. While an increase in oxidative stress at 20% oxygen compared to 5% is widely accepted to occur in the early embryo, there is still debate as to whether the same is true for the COC during IVM, and species-specific differences might exist. Bermejo-Álvarez et al., (2010) reported an increase in developmental competence in bovine oocytes after IVM at 5% oxygen compared to 20%, however when expression of genes related to oxidative response (*GPX1* and *MNSOD*) was evaluated, no differences were found. The authors did note however that this does not rule out a role of oxidative stress at atmospheric oxygen concentration, as the response could be post-transcriptional. Moreover, gene expression results do not necessarily indicate changes in protein content or enzyme activity.

A higher number of COCs depleted all available glucose at 5% oxygen compared to the same pyruvate concentration at 21% (26% depleted in Group 5C vs. 2.9% in Group 21C). While there are no similar studies evaluating glucose consumption directly, several studies in the cow, mouse, human and buffalo have reported increased expression of glycolytic pathway genes in COCs matured at 5% oxygen (Bermejo-Álvarez et al., 2010; Hashimoto et al., 2000; Kang et al., 2011; Kumar et

al., 2015). It has been shown that such changes in gene expression are regulated by the oxygen sensitive hypoxia-inducing factors (HIFs; Kind et al., 2015; Thompson et al., 2015), a pathway also utilised by cancer cells as a means of switching from oxidative to glycolytic metabolism (Semenza, 2010). This adaptation is thought to limit oxidative stress and enhance survival in hypoxic conditions. Interestingly, *in vivo* this is proposed to be further mediated by the presence of haemoglobin in cumulus cells which acts to bind and lower the oxygen concentration thereby promoting HIF activation (Brown et al., 2015). When the glucose-depleting COCs were removed from analysis, there was no relationship between oxygen consumption and glucose consumption, moreover there was no change in glycolytic index. A more definitive measure would be to record oxygen consumption and mitochondrial function directly at both oxygen concentrations in order to conclude if there was a shift between glycolysis and oxidative phosphorylation. Unfortunately, the present method involving the Seahorse XFp analyser does not allow measurements at low oxygen concentrations. There are no other reports of equine IVM at low oxygen concentration and as developmental competence was not evaluated in the present study, this remains an interesting area for further research.

In this study, the aim was to adjust experimental conditions in light of the high rate of glucose depletion (in 18-24% of COCs) reported in the pilot study; Chapter 3. Increasing the drop size to 15 μ l (from 10 μ l previously) and trimming excess granulosa cells successfully reduced the proportion of COCs depleting glucose in the standard conditions (Group 21C) to 2.9% (1/35). This has both clinical and experimental implications. Clinically, these modifications ensure that glucose availability is not an impediment to full developmental potential, and experimentally, the analysis of substrate flux may be determined more accurately under conditions when glucose availability is not a limiting factor.

As shown in Chapter 3, glucose consumption and lactate production were positively correlated to DNA content of the COC (a proxy measurement of cumulus cell numbers). In contrast, pyruvate production was independent of DNA content, and

the final concentration of pyruvate in the medium was 0.2-0.3 mM after the 30 hour IVM for all COCs. This is interesting as O'Fallon and Wright, (1974) reported an absolute medium concentration of 0.3 mM to be protective against ROS in the early murine embryo. Leese and Lenton, (1990) proposed an elegant model of pyruvate homeostasis within the human follicle in which the pyruvate concentration was a reflection of the lactate dehydrogenase reaction equilibrium between lactate and pyruvate to regulate the pH. However, as the culture medium was already buffered, the definitive reason for this observation remains unclear.

Addition of pyruvate (0.15 mM) increased glucose consumption rather than acting to spare it. This was unexpected given that the COCs seemed to prioritise the production of pyruvate, which presumably was derived directly from glycolysis or by conversion from lactate, the source of which could also have been glucose as there was minimal lactate in the starting medium (< 1mM from FBS). Also interesting was that addition of pyruvate (up to 0.5 mM) did not alter oxygen consumption or mitochondrial function of COCs. Taken together, these findings suggest that the presence of pyruvate resulted in a higher rate of glycolysis, but at the same proportion in relation to other pathways, as lactate : glucose ratio remained constant, and these treatments had a similar rate of oxidative phosphorylation. This lack of increase in OXPHOS is not surprising as even in standard conditions (Group 21C) mitochondria were working at close to maximal rate as per the very low spare capacity at the end of IVM (< 10%).

As found in the pilot study in Chapter 3, temporal differences were observed over the course of IVM and with increased numbers and replicates these differences became significant. In agreement with the findings of Alm and Hinrichs (1996) that GVBD and progression to MII occurred between 12-16 hours, we observed a significant increase in glucose consumption between 10-20 hours coinciding with the increased metabolic demands at this time. To confirm that the metabolism measured was a function of events related to the COC itself and driven by the oocyte, granulosa cells were cultured alone and compared to intact COCs. It was reassuring to find that there was no variation in glucose and lactate metabolism

over time in granulosa cells cultured alone, verifying that the oocyte was influencing energy requirements in the COCs. It is also possible that the more highly differentiated corona radiata or cumulus cells (not included in the granulosa only samples in the current study) also influence temporal changes in glucose metabolism as when bovine oocyte-ectomised COCs were compared to intact COCs, no differences in glucose metabolism were observed (Sutton et al., 2003). Interestingly, the decreased pyruvate production pattern at the end of the maturation period when granulosa cells were cultured alone was opposite to that of COCs, which demonstrated increased pyruvate production during this time period of 20-30 hours.

Oocytes undergoing meiosis have an increased requirement for pyruvate (Downs et al., 2002) which may explain the decreased production of pyruvate during 0-20 hours and its subsequent increase in production when it was no longer required at the end of maturation, when the oocyte had reached metaphase II. Another possible explanation for increased pyruvate production at the end of IVM, could relate to its antioxidant capacity given the decreased mitochondrial spare capacity at the end of IVM reported in Chapter 3. The oocyte is known to have several hundred thousand mitochondria compared to only a few hundred in a granulosa cell (Collado-Fernandez et al., 2012). It has been hypothesized that this low individual activity minimises oxidative stress (Dumollard et al., 2007), but if metabolic activity is high as in these COCs near maximal respiration, it may mean that more ROS are produced placing the COC in an oxidative state and therefore requiring more pyruvate to limit oxidative damage.

Gonadotropin addition to IVM medium has multiple actions, including mediating cumulus expansion and driving meiotic resumption (reviewed by Rodriguez and Farin, 2004). The proposed mechanism is an increase in intracellular communication brought about by elevated connexin 43 levels through activation of protein kinase A (PKA) (Sommersberg et al., 2000), events shown to cause an increase in glucose uptake and lactate production by the cumulus cells (2.7 fold increase in glycolysis; Downs and Utecht, 1999). In the mouse and the cow, addition of FSH also

upregulated expression of glucose transport proteins such as GLUT 4 and glycolytic enzymes in cumulus cells, (Roberts et al., 2004; Sutton-McDowall et al., 2004). Increased glucose consumption in response to FSH can further be attributed to increased HBP flux in order to synthesize hyaluronic acid essential for cumulus expansion (Sutton-McDowall et al., 2010). In the present study, while the presence of FSH resulted in an increase in glucose consumption and lactate production, only the increase in lactate production at 10-30 hours was significant ($p < 0.05$). As such, there was no fall in glycolytic index attributable to FSH to account for the presumed increase in HBP activity in the presence of FSH. Unexpectedly, the presence of FSH also increased pyruvate production at 10-30 hours. This could potentially be a regulatory response to stabilise the lactate : pyruvate ratio and hence the pH however it is not easily explained at this time.

Conclusions

The main findings that emerged from the experiments carried out in this chapter are as follows (i) a strategy (reduction of cumulus mass and increase in droplet size) was confirmed to reduce the proportion of COCs depleting available glucose during IVM in clinical conditions (ii) measures of equine COC metabolism, i.e. glucose consumption and lactate/ pyruvate production are driven by the oocyte, as they differed between COC and isolated granulosa cells (iii) FSH stimulates lactate and pyruvate production in the COC (iv) low oxygen and presence of 0.15 mM pyruvate during IVM results in a decreased glycolytic index and decreased pyruvate production suggestive of a decreased oxidative state of the COC, (v) addition of pyruvate did not spare glucose consumption or ameliorate the issue of low mitochondrial spare capacity at the end of IVM.

Chapter 5: The effect of increased glucose concentration during IVM on equine oocyte developmental markers, glucose metabolism and mitochondrial function.

5.1 Introduction

The potential influence of the periconceptual environment on oocyte quality, oocyte developmental competence and future health of the offspring is well established (Fleming et al., 2018). Assisted conception technologies aim to mimic the periconceptual environment, so it is important to make sure they are optimised to the best of our knowledge. In the horse, there is a paucity of species-specific IVM optimisation and many differences exist in protocols between laboratories. It is as yet unknown what effect many of these procedural differences have on oocyte developmental potential. The two most commonly used media for equine IVM are TCM-199, originally designed for chick embryo fibroblasts (i.e. somatic cells) and first reported in the horse by Zhang et al., (1989) and DMEM/F12, also a complete tissue culture medium, first described for use in equine IVM by Galli *et al.*, (2007). M199 supplies glucose at 5.6 mM, i.e. near the range found in equine follicular fluid and serum while DMEM/F12 contains a supraphysiological concentration of glucose at 17 mM. However, both systems have produced viable embryos and healthy offspring after ICSI and embryo culture (Galli et al., 2007; Hinrichs et al., 2014, 2007; Smits et al., 2010).

In the horse, serum glucose levels are controlled between 4.9-6.2 mM. Unlike other species, insulin resistance associated with diseases such as obesity and metabolic syndrome is characterised by secondary hyperinsulinaemia and as such, hyperglycaemia does not occur. In contrast, pancreatic exhaustion occurs in human insulin resistance leading to decreased insulin secretion and hyperglycaemia as in Type-2 diabetes (Morgan et al., 2015). In fact, clinical hyperglycaemia in the horse is only found at the end stage of diseases such as sepsis when death is imminent (Hollis et al., 2007). Thus, the equine COC and embryo would never experience hyperglycaemia *in vivo*. Additionally, the follicular fluid glucose concentration is lower than that of serum and further decreases as ovulation approaches ($4.72 \pm$

0.29 mM 24 hours after dominant follicle selection [largest follicle 20-25 mm] falling to 3.24 ± 0.09 mM 33 hours after induction of ovulation; (Collins et al., 1997; Gerard et al., 2000; Gérard et al., 2002).

In humans, the detrimental effect of hyperglycaemia during pregnancy is well described and adverse clinical outcomes include increased risk of abortion, pre-eclampsia, pre- term birth and neonatal/childhood illness (Frank et al., 2014). The risk of congenital defects is also up to 20 times higher in children born to mothers experiencing hyperglycaemia during pregnancy than in those born to normoglycaemic mothers (Becerra et al., 1990; Bell et al., 2012; Casson et al., 1997; Eriksen et al., 2017). Notably, this risk is significantly lowered only if glycaemic control is instituted before contraception is discontinued, pointing to the follicular environment and the periconception period as a critical time window (Pearson et al., 2007). In this regard, work in mice has demonstrated that at the cellular level, hyperglycaemia during oocyte maturation results in delayed meiotic kinetics, increased apoptosis in granulosa cells, decreased oocyte ATP content, aberrant glucose metabolism, changes in mitochondrial distribution and morphology and decreased oocyte-cumulus cell communication (Chang et al., 2005; Colton et al., 2003; Frank et al., 2014; Ratchford et al., 2008; Wang et al., 2010)

Given the range of detrimental effects of increased glucose in other species, it is striking that good blastocyst rates and clinical foal production have been reported by equine laboratories performing IVM in both M199 and DMEM/F12 based medium (5.6 mM and 17 mM glucose respectively; Galli et al., 2007; Hinrichs et al., 2014, 2007; Smits et al., 2010). In the single study in which these media have been compared, it was reported that DMEM/F12 was superior to M199-based IVM medium when considering subsequent blastocyst development (26.4% [37/140] for DMEM/F12 vs. 12% [23/191] for M199; Galli *et al.*, 2007). However, in another laboratory, M199-based IVM medium has produced the highest reported blastocyst rates (up to 46%) in numerous reports (Hinrichs et al., 2005; Jacobson et al., 2010; Ribeiro et al., 2008; Salgado et al., 2018). Notably, equine *in vitro* embryo culture often also involves supraphysiological levels of glucose (17 mM, DMEM/F12).

Research to date suggests that not only does this concentration of glucose not lead to embryo developmental arrest, as it would in other species such as the cow, but the equine embryo seems to thrive, as demonstrated by 46% blastocyst development at 20 mM glucose; Choi et al., 2015, Choi et al., 2004).

The known mechanisms by which hyperglycaemia causes downstream effects in oocytes and embryos are largely related to carbohydrate metabolism and are similar to those reported in somatic cells. The hexosamine biosynthesis pathway (HBP) has received particular attention as a potential central mediator of glucotoxicity in COCs, embryos and somatic cells due to its end product UDP-N-Acetylglucosamine resulting in excessive β -O linked glycosylation of proteins, (Figure 5.13; Pantaleon et al., 2010; Sutton-McDowall et al., 2010; Wong et al., 2015) as has overproduction of reactive oxygen species (ROS) due to increased oxidative damage and aberrant glucose utilisation (Rolo and Palmeira, 2006). To date, the consequences of supraphysiological glucose concentration during equine IVM are unknown. The establishment of methods, as reported in previous chapters, for investigation of parameters such as glucose, lactate and pyruvate measurement in addition to oxygen consumption measurements and mitochondrial efficiency testing in equine COCs, allows the effect of glucose concentration on COC carbohydrate metabolism to now be explored.

A further tool to investigate the downstream effects of changes to the IVM environment is to evaluate developmental potential (blastocyst formation after ICSI) and gene expression in the resulting blastocysts. In other species, there are many studies demonstrating a clear negative effect on blastocyst development associated with increased glucose concentration during the period of preimplantation embryo development (Frank et al., 2014; Fraser et al., 2007; Moley et al., 1998). However, fewer studies demonstrating a reduction in blastocyst development attributable specifically to supraphysiological glucose during the period of IVM are available; these have been carried out in mice, cattle and buffalo (Frank et al., 2013; Hashimoto et al., 2000; Kumar et al., 2012; Lea et al., 1996). When considering aberrations in blastocyst gene expression, the situation is similar.

Hyperglycaemia⁴ during the period of early embryo development is associated with decreased expression of insulin and IGF-1 receptors in rabbit blastocysts (Ramin et al., 2010), decreased glucose transporter expression and increased expression of apoptosis related genes in mouse embryos (Moley et al., 1998; Moley et al., 1998) and increase in expression of genes related to the Warburg effect i.e. increased glycolysis and decreased OXPHOS in cattle embryos (Cagnone et al., 2012). To the best of my knowledge studies investigating blastocyst gene expression as a result of the isolated effect of glucose during IVM have not been carried out in any species. Evaluating gene expression in blastocysts can be extremely informative; both for understanding metabolic pathways and the way they are regulated at the transcription level but also as a general marker of developmental competence.

The aim of this study was to measure expression of genes for factors in key metabolic pathways and related enzymes of carbohydrate metabolism known to be affected by hyperglycaemia. These include; glycolysis (phosphofructokinase [PFK] and lactate dehydrogenase [LDHA]), pentose phosphate pathway (glucose-6-phosphate dehydrogenase [G6PD]) and glucose transport (solute carrier family 2 member 1 [SLC2A1; formerly GLUT1]). Since hyperglycaemia also affects lipid metabolism via the glucose: fatty acid cycle (De Bie et al., 2017; Wong et al., 2015), the relative expression of the gene encoding carnitine palmitoyltransferase 1A (CPT1A), a regulatory enzyme in β -oxidation and fatty acid transport, was also evaluated. As oxidative stress is known to be an additional regulator of the response to hyperglycaemia, relative expression of genes encoding glutathione peroxidase 1 (GPX1) and mitochondrial transcription factor A (TFAM) were examined. GPX1 is a potent antioxidant enzyme, central in defence against H_2O_2 , and abundant in oocytes and embryos (Agarwal et al., 2012). TFAM is involved in mitochondrial biogenesis and mtDNA transcription and translation (Itami et al., 2015), and is known to be affected by hyperglycaemia in human somatic cells and cumulus cells (Palmeira et al., 2007; Suarez et al., 2008; Wang et al., 2010).

⁴ For brevity, the term hyperglycaemia is used throughout this thesis to connote supraphysiological glucose concentrations both *in vivo* and in media *in vitro*.

In addition to functional changes in carbohydrate metabolism, evaluation of expression of pluripotency marker genes (*POU5F1*, *SOX2* and *NANOG*) and genes for markers of trophectoderm differentiation (*CDX2* and *TEAD4*) can give insight into blastocyst quality and timely development (Filliers et al., 2012; Li et al., 2005; Purpera et al., 2009), and were thus measured. Successful embryo development requires a series of cell lineage differentiation events, the first being differentiation of cells into trophectoderm (TE) and inner cell mass (ICM) cells (containing endoderm and future epiblast cells; Kuijk et al., 2012; Schrode et al., 2014), followed by spatial segregation of the latter two cell types into the hypoblast and epiblast. These events are tightly controlled by temporal expression of developmental genes, the sequence of which is partially species specific (Choi et al., 2009; Filliers et al., 2012; Kuijk et al., 2008; Paris et al., 2012).

POU5F1 (*OCT4*), is a regulatory transcription factor essential in cell lineage differentiation and maintenance of pluripotency (Ovitt and Scholer, 1998; Wu and Schöler, 2014). *POU5F1* is both maternally and zygotically expressed but as embryo development progresses to the blastocyst stage it is increasingly confined to the ICM in the mouse and human, however in the pig and cow there appears to be expression in both the ICM and TE until after the blastocyst stage (Kimber et al., 2008; Kirchhof et al., 2000). *SOX2*, acts with *POU5F1* to maintain pluripotency in the ICM and to regulate *NANOG*. *NANOG* is an epiblast-specific ICM transcription factor downstream of *POU5F1/SOX2*, and in the absence of *NANOG*, ICM cells become GATA-6 positive primitive endoderm (Mitsui et al., 2003). *CDX2* is a TE specific transcription factor which acts in a reciprocal manner with *POU5F1* to determine ICM/TE fate. In the absence of *CDX2* there is no downregulation of *POU5F1* and therefore no TE formation (Strumpf et al., 2005). As such, *CDX2* expression increases over the course of early embryo development as the TE forms and the number of TE cells increases (Filliers et al., 2012; Paris et al., 2011). Upstream of *CDX2*, *TEAD4*, controls *CDX2* expression after the morula stage; thus in the absence of *TEAD4* there is no upregulation of *CDX2* and hence all blastomeres remain *POU5F1* positive (Nishioka et al., 2008). Given their importance in initial differentiation and ICM formation *POU5F1* and *SOX2* have been suggested as

markers of developmental potential (Filliers et al., 2012; Kimber et al., 2008; Li et al., 2005).

In the horse, studies on blastocyst gene expression are scarce. Differences have been reported in both quantitative and spatial expression of *POU5F1* and *CDX2* between IVP and *in vivo* embryos (Choi et al., 2009; Paris et al., 2012). A study by Smits et al., (2011) demonstrated downregulation of five developmentally important genes in Day-9 IVP blastocysts compared to Day-7 *in vivo* blastocysts, while Hendriks et al., (2015) found that expression of mitochondrial replication gene *mtPOLB* was upregulated and the gene coding antioxidant enzyme, *GPX3* downregulated in Day-8 IVP blastocysts compared to Day-7 *in vivo* blastocysts. However, these comparisons may not be meaningful, as they were made between embryos at very different stages of development (morula/early blastocyst for IVP embryos, vs. expanded blastocyst for *in vivo* embryos). Using immunohistochemistry, Choi et al., (2015) reported differences in cell lineage allocation genes at the protein level attributable to glucose concentration during early embryo culture while Desmarais et al., (2011) demonstrated differences in *GATA-6* allocation between *in vivo*, nuclear transfer and parthenogenetic equine embryos. Differential antibody staining reveals that IVP equine embryos resemble Day-6 *in vivo* embryos, as there is no distinct formation of an ICM; rather the epiblast and endoderm cells are distributed in a network within the trophoblast cavity (Bruyas et al., 1993; Hinrichs et al., 2007; Tremoleda et al., 2003). Additionally, formation of hypoblast and epiblast does not appear to occur in horse embryos; rather the endodermal cells of a central unorganized cell network seed directly onto the inner surface of the trophoblast (Choi et al., 2015; Enders et al., 1993).

Summary

The combined approach of investigating COC carbohydrate metabolism and blastocyst gene expression in response to hyperglycaemia during IVM may provide insights into the mechanism by which, unlike oocytes and embryos of other species, the equine oocyte can tolerate such high levels of glucose. The experimental set up of individual COC culture and the Primovision™ time-lapse system, uniquely allows associations between COC carbohydrate metabolism during IVM, morphokinetics during embryo development and gene expression in the resulting blastocysts to be explored.

5.2 Objectives

1. Evaluate the impact of increased medium glucose concentration (17 mM vs. 5.6 mM) during IVM on:
 - a. Oocyte maturation, embryo cleavage and blastocyst rates after ICSI (Experiment 5.1; Study 1)
 - b. Morphokinetics of early embryo development using Primovision™ time-lapse imaging (Experiment 5.1; Study 2).
 - c. Glucose, lactate and pyruvate metabolism of COCs during IVM (Experiment 5.1; Study 3).
 - d. Basal oxygen consumption rate and mitochondrial function of COCs at the completion of IVM (Experiment 5.2).
 - e. Expression of genes related to metabolism, oxidative stress and developmental competence in the blastocysts.
 - I. Measure the relative expression of genes for factors associated with glucose metabolism (*G6PD*, *PFKM* and *LDHA*), oxidative stress (*GPX1* and *TFAM*), glucose transport (*SLC2A1*), fatty acid transport (*CPT1A*), pluripotency marker genes (*POU5F1*, *SOX2* and *NANOG*) and trophectoderm markers (*CDX2* and *TEAD4*) in IVP blastocysts (Experiment 5.3; Study 1)
 - II. Explore associations between the relative expression of the above genes. (Experiment 5.3; Study 2A)
 - III. Explore the association between the relative expression of the above genes in the blastocysts and the corresponding COC metabolism during IVM. (Experiment 5.3; Study 2B)
 - IV. Explore the association between the relative expression of the genes in (i) and blastocyst morphokinetics during development (Experiment 5.3; Study 2C).

5.3 Materials and Methods

General methods

COCs were recovered from abattoir-derived ovaries and classified as compact (Cp), expanded (Ex) or corona radiata-only as previously described in Section 2.1.1 The COCs were held overnight at room temperature (Section 2.1.2). When removed from the holding vial, compact (Cp) and expanded (Ex) COCs were divided equally between the different conditions, so that for each collection day, each maturation condition contained equal numbers of each cumulus type.

Experiment 5.1

Culture for IVM was carried out in individual 10- μ l droplets under oil of either Control Maturation Medium (5.6 mM glucose; M199 with Earle's salts, 10% FBS, 25 μ g/ml gentamicin with 5 mU/ml FSH) or High glucose Maturation Medium (17 mM Glucose; M199 with Earle's salts and 11.4 mM added glucose, 10% FBS, 25 μ g/ml gentamicin with 5 mU/ml FSH) and conducted at 5% CO₂ in air. High glucose medium (17 mM) was prepared by increasing the concentration of stock control media by the addition of 1.026 g embryo grade glucose (Sigma) to 500 ml M199 media. This raised the osmolarity by approximately 11.4 mOSM, which required approximately 3% dilution to ensure it was iso-osmolar with the control medium (290-300 mOsm). This in turn lowered the final glucose concentration to 16.5 mM, rounded to 17 mM throughout. Twelve droplets were placed in each dish; of these, three medium droplets remained empty and were used as controls. Dishes were continuously incubated for the entire IVM period (30 hours; See Section 2.2.4 for full description).

After the maturation period was completed, COCs were denuded and classified as Metaphase II, intact or degenerated as previously described in Section 2.1.4. All denuded cumulus cells from each COC were placed in a 96-well plate and kept at -20° C until later DNA quantification analysis. Spent media were frozen at -80° C and analyses of thawed samples carried out as previously described in Section 2.2

Metaphase II oocytes underwent ICSI and subsequent embryo culture as described in Section 2.1.6. Injected oocytes were continuously monitored using a Primovision™ Time–Lapse System. Zona pellucida thickness and oocyte diameter were measured on the first frame by taking the mean of at least two measurements. Time of cytoplasmic extrusion, first cleavage and blastocyst formation was recorded for each injected oocyte where visualisation allowed (this was complicated by the dark, opaque nature of the horse oocyte/embryo cytoplasm and excess movement due to adherent cumulus cells). Diameter of the embryo at the time of blastocyst formation was recorded. Cytoplasmic extrusion was defined as the time cytoplasmic contents were first extruded from the oolema. Time of cleavage was defined as the time of the first visually identifiable indentation of the oolema before a visualized cleavage. Embryos were classified as blastocysts on first appearance of apparent formation of a central fluid-filled cavity (blastulation). All annotations were performed by a single operator, blind as to oocyte treatment group.

Experiment 5.2

In vitro maturation was carried out directly in a Seahorse plate. COCs were matured in groups of 3 COCs in 180 µl of either control maturation medium or high glucose maturation medium at 5% CO₂ in air. (As per Experiment 1; full details of maturation in Seahorse plate in Section 2.4.3). The Seahorse plate was removed from the incubator and placed in the Seahorse analyser 28 hours after initiation of IVM (completion of IVM; IVM + 28 hours). After calibration, three basal oxygen consumption rate readings (pmolO₂/well/min) were recorded before a series of respiratory inhibitors were added sequentially to each well to achieve the following final concentrations; 1) Oligomycin [1µM] 2) FCCP [5µM] 3) Antimycin/Rotenone [2.5µM]. *See Section 2.4.3 for full description of method. After the readings were obtained, COCs were placed into a plate and frozen for later DNA quantification.

Experiment 5.3

All blastocysts produced were used experimentally. Lysis and polyA amplification, cDNA quantification and storage were carried out as previously described in Section 2.5.1. A total of twelve genes of interest were investigated and Taqman® probe based primers designed within 300 bp of the polyA tail at the 3' UTR (Primerdesign LTD; Table 5.1). All primers were optimised and tested using amplified cDNA from an equine IVP blastocyst by Primerdesign LTD.

Name	Accession number	Sense primer	Antisense primer
G6PD	XM_001492232	CTTGCCACTAGGAAAGTA GAAGC	AGGCAGGTCTGTTTGTGG ATT
PFKM	NM_001081922	TGGAAGCTCCTTTTAGGT AGAATTTAT	AATCTCGCAGTGGCACTA GAGG
CPT1A	NM_001081808	TTCCTTCCATCCAGGGTTG G	GACCTGCCCAGAAGTAGT AGG
GPX1	NM_001166479	GAACGCCTGATGTTAAGG AGAAT	TTATTAGTGAGAAGCCGT GGTCT
TFAM	XM_001503382.3	ATCTTGGGAAGAACAAAT GATTGAAG	TGTCTATCTACTGTGAACA TAACTCAA
LDHA	NM_001144880	GATGCGTGTTTACTCTGTG TGAT	TCTCAAGATGTGACTGACT GAAGA
SLC2A1	NM_001163971	GAGGTCAGGCTCCATTAG GATT	CAACTGGTCTCAGGCAAG GA
POU5F1	XM_01473467	GGATCAGGTTCTTCATTCA CTAAGA	AATCAAGAGCATCATTGA ACTTCAC
SOX 2	XM_00336334	GTAGTTGTATTTTAAAGAT TCGGCTCT	ACCTGTTATAAGGATGGT ATTAGTTCA
NANOG	XM_014740545	GCTGTGGAGAGGACTTCC TG	TTGCTGTGTTGCGTTATGG C
CDX-2	XM_014731940	GGCTGAATGTATGTCAGT GCTATAA	TGGATGTTACACAGACCA ACAAC
TEAD4	XM_014740495.1	AAGCTCATGGATGTCTAA TATTCAATT	GTGTGTGGCAGACTTTAC TGAA

Table 5.1: Genes and primer sequences for genes of interest in blastocysts.

The qPCR reactions were made up as follows and plated in triplicate: 5 µl cDNA (5 ng), 1 µl primer, 10 µl PrecisionPlus mastermix (Primerdesign LTD) and 4 µl PCR water. Amplification was performed on a Biorad CFX using the following protocol; initial enzyme denaturation of 2 minutes at 95 °C then 50 cycles of 15 seconds at 95 °C and 60 seconds at 60 °C. Data were collected through the FAM channel at the final stage. Reference genes (*RPL32* and *SRP14*; Section 2.5.1) were also run for each sample on each plate. All blastocysts and all genes were plated on a single plate.

Statistical analysis

For Experiment 5.1, Study 1, parameters Metaphase II (Y/N), cleavage (Y/N) and blastocyst (Y/N) were set as binary outcomes and univariate analyses using the Chi-square test were performed to explore associations related to IVM condition. Fisher's exact test was used when values in a cell were less than five. In Study 2, morphokinetic parameters were noted for each injected oocyte and compared between IVM conditions using Student's t-test. In Study 3, the primary outcome variables were glucose consumption, lactate and pyruvate production (expressed as pmol/ngDNA/hour). Student's t-test was used to explore to associations related to IVM condition.

In Experiment 5.2, data were expressed as pmolO₂/COC/hour or pmolO₂/ngDNA/hour. Spare capacity and proton leak were corrected to zero if a negative value was obtained. Student's t-test was used to compare readings between experimental IVM conditions after arcsine transformation of percentage data.

Experiment 5.3, Study 1: The mean Ct value from each technical triplicate was calculated for each sample and retained if variation was <1 Ct. Δ ct was then calculated for each sample by subtracting the Ct value of the reference gene of that sample from the Ct value of the gene of interest. Relative expression ($2^{-\Delta\text{Ct}}$) was then presented for each blastocyst (Schmittgen and Livak, 2008). In Experiment 5.3, Study 2A: Pairwise regression comparisons were performed for each gene (90

regression comparisons) in order to determine if any significant associations existed. Experiment 5.3, Study 2B and 2C: Relative gene expression for each gene in each blastocyst was analysed against the corresponding COC glucose consumption, lactate and pyruvate production rates (all in pmol/ngDNA/hour) and the ratio of lactate production to glucose consumption during IVM (30 comparisons; Study 2B). Relative gene expression was also analysed against morphokinetic indices during development for each blastocyst (time of blastulation and diameter at blastulation; 20 comparisons; Study 2C).

For full details on statistical analyses including criteria for data cleaning and replicates see Section 2.5.

5.4 Results

5.4.1 Experiment 5.1

One hundred and seventy six oocytes were evaluated over five replicates. Of 55 MII oocytes in the control (5.6 mM glucose) group, six were not subjected to ICSI (two used for other studies (cDNA) and four rejected due to adherent cumulus cells). Of 45 MII in the high (17 mM glucose) group, 11 were not injected (two used for other studies (cDNA) and nine rejected due to adherent cumulus cells; see Table 5.2).

Study 1- Effect of glucose concentration during IVM on development post ICSI

The concentration of glucose added to the medium during IVM did not influence maturation, cleavage or blastocyst rate (Table 5.2). Blastocyst rates were low overall, however.

	IVM 5.6 mM Glucose	IVM 17 mM Glucose	P
Maturation rate	62.5% (55/88)	51% (45/88)	0.13
Cleavage rate	75.5% (37/49)	76.5% (26/34)	0.92
Blastocyst rate	8.2% (4/49)	2.9% (1/34)	0.4

Table 5.2: Effect of glucose concentration during IVM on maturation, cleavage and blastocyst rate post ICSI.

Study 2- Effect of glucose concentration during IVM on morphokinetics of early embryo development

All injected MII oocytes were analysed (n = 83), however data was lost for 2 COCs in the 17 mM glucose group due to technical issues, therefore for final analysis n = 81. Cytoplasmic extrusion and cleavage determination was possible for 37 and 48 oocytes respectively. As there were only five blastocysts, the effect of glucose on blastocyst morphology/morphokinetics could not be evaluated. There were no differences attributable to glucose concentration during IVM on any of the four morphokinetic parameters measured (Figure 5.1).

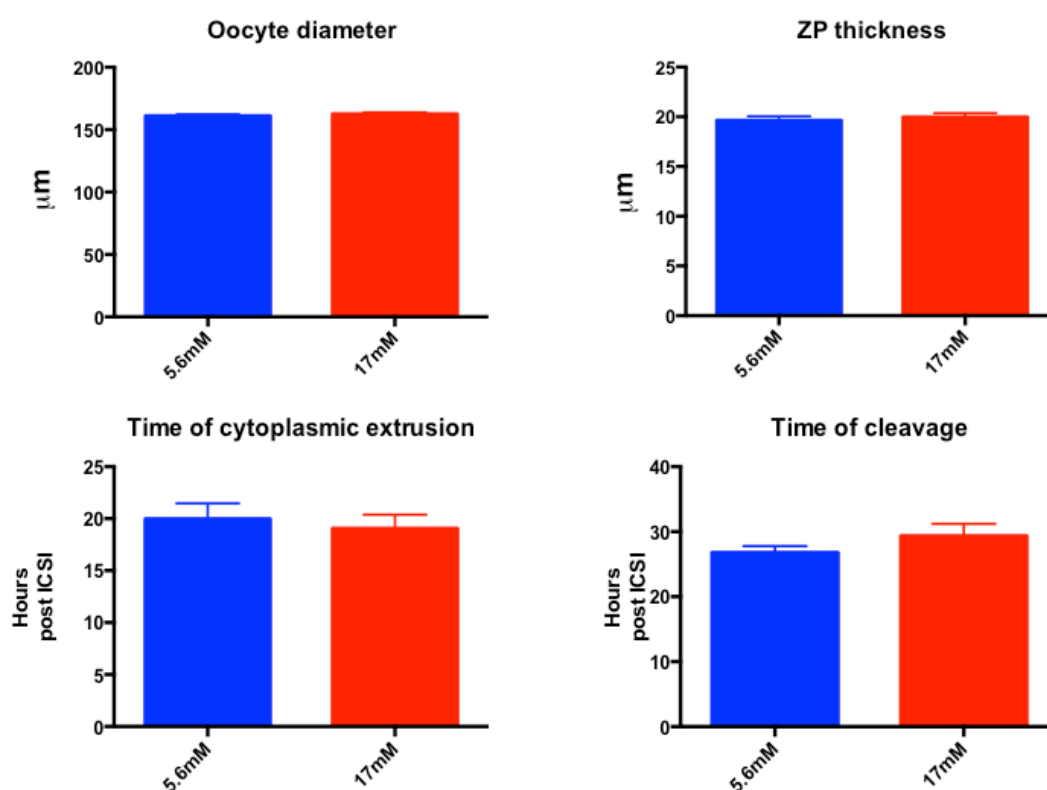


Figure 5.1: Effect of glucose concentration (5.6 mM [BLUE] and 17 mM [RED]) during IVM on morphokinetic parameters measured using the Primovision™ Time-lapse system. Bars represent mean \pm SEM.

For descriptive purposes, time of cytoplasmic extrusion, cleavage and blastocyst formation (range 165.2-224.6 hours [mean 183 hours \pm 11 SEM]) is shown in Figure 5.2 for each blastocyst. All blastocysts were lysed for mRNA extraction and cDNA synthesis at 21.6-31.6 hours post blastulation (mean 25.9 hours \pm 1.7 SEM).

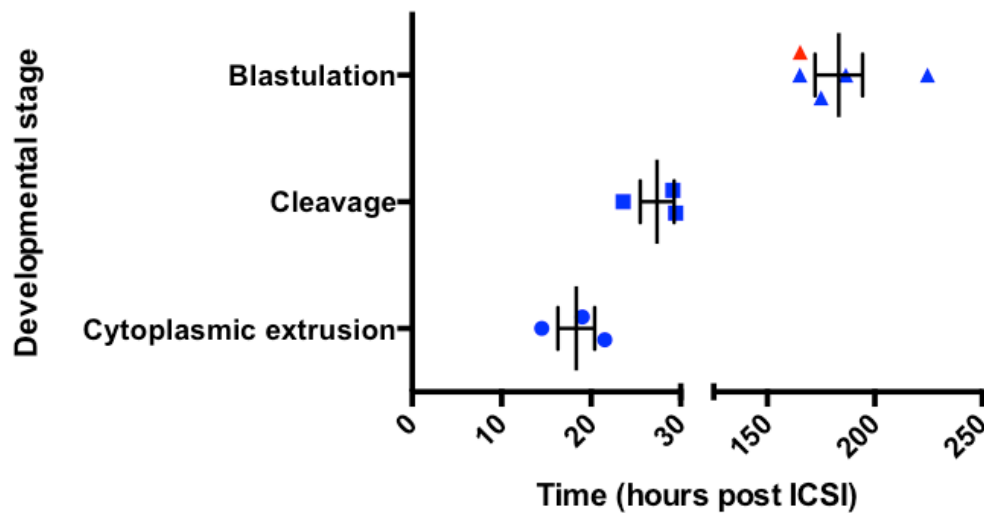


Figure 5.2: Developmental timings for each of the five blastocysts. Mean \pm SEM are presented. Blastocysts from the 5.6 mM glucose IVM group are in BLUE and the single blastocyst from the 17 mM IVM group is in RED. Detection of cleavage and fragmentation timing was only possible for 3/5 blastocysts due to loss of focus of the Primovision camera as a result of excess zygote movement.

Study 3- Effect of glucose concentration during IVM on COC metabolism.

Metabolic data were analysed for all injected MII oocytes and those used for cDNA with the following exceptions; 5.6 mM glucose group, n = 1 lost due to data cleaning (final analyses n = 50); 17 mM glucose group, n = 2 lost due to data cleaning and n=4 lost due to technical issues (final analyses n = 30). Of COCs in the control 5.6 mM glucose group, 18% (9/50) depleted all available glucose, vs. 0/30 in the 17 mM glucose. One of the nine COCs that depleted all available glucose went on to form a blastocyst. When evaluating those that did not deplete available glucose and therefore for which glucose was not limiting, glucose concentration during IVM did not affect glucose consumption, lactate/pyruvate production or lactate : glucose ratio (Figure 5.3).

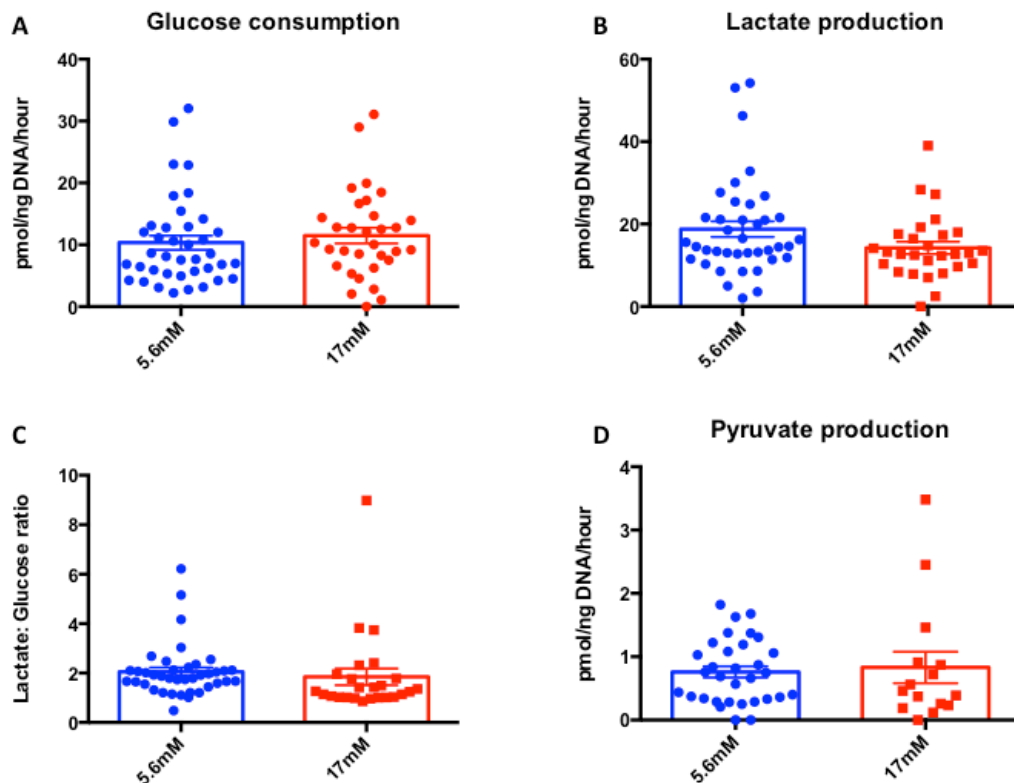


Figure 5.3: Effect of glucose concentration (5.6 mM vs. 17 mM) during IVM on glucose consumption, lactate production, lactate : glucose ratio and pyruvate production. Bars represent mean \pm SEM. Data points represent individual COCs

5.4.2 Experiment 5.2: Effect of glucose concentration during IVM on OCR and mitochondrial function.

Fifty-four COCs were used for this experiment across three replicates (27 in each condition). Thirty COCs had compact cumulus and 24 COCs had expanded cumulus. Data from three expanded COCs in each condition were excluded from subsequent analysis due to biologically implausible OCR readings (See Table 5.3 for final numbers available for analysis)

	IVM 5.6 mM Glucose	IVM 17 mM Glucose
Cp	15	15
Ex	9	9
Total	24	24

Table 5.3: Final numbers of COCs analysed for Experiment 5.2. Cp = COCs with compact cumulus and Ex = COCs with expanded cumulus.

There were no differences attributable to cumulus classification within experimental condition ($p > 0.5$) therefore Cp and Ex were treated as one group for further analysis. The basal OCR showed a non-statistically significant trend towards being lower in the 17-mM glucose group (Figure 5.4). When mitochondrial function was evaluated, the proportion of OCR coupled to ATP production was lower in the 17-mM glucose group ($42 \pm 5.3\%$ vs. $62 \pm 4.8\%$ in the 5.6-mM glucose group; $p = 0.01$) and non-mitochondrial OCR was higher ($48 \pm 6\%$ in 17-mM vs. $26 \pm 6.1\%$ in 5.6-mM; $p = 0.02$; Figure 5.5).

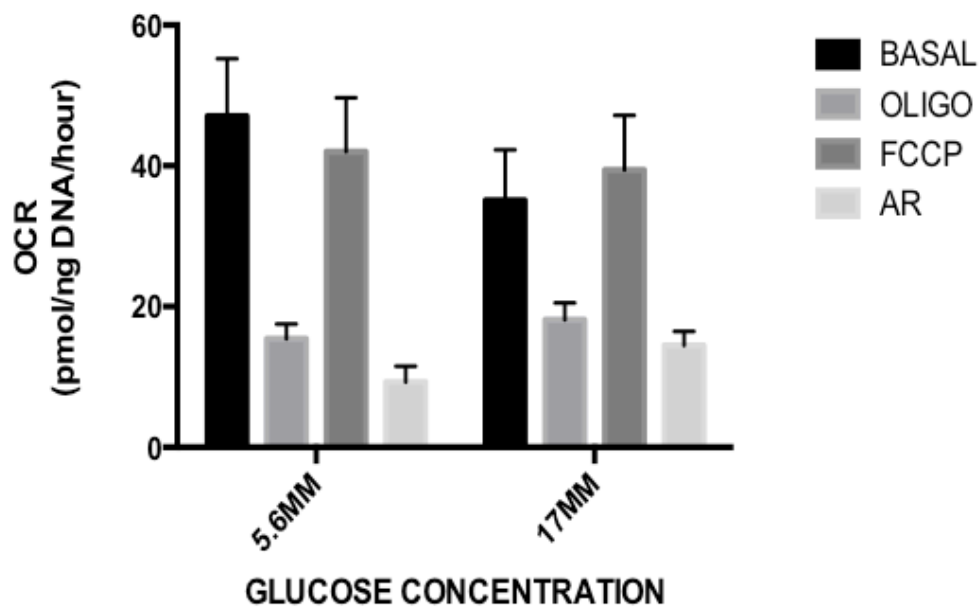


Figure 5.4: Effect of glucose concentration during IVM on oxygen consumption rate (OCR) after sequential addition of respiratory inhibitors to evaluate mitochondrial function ($n = 48$). Each bar represents the mean OCR after addition of the inhibitor stated (\pm SEM). Abbreviations as follows: OLIGO: Oligomycin; AR: Antimycin/rotenone.

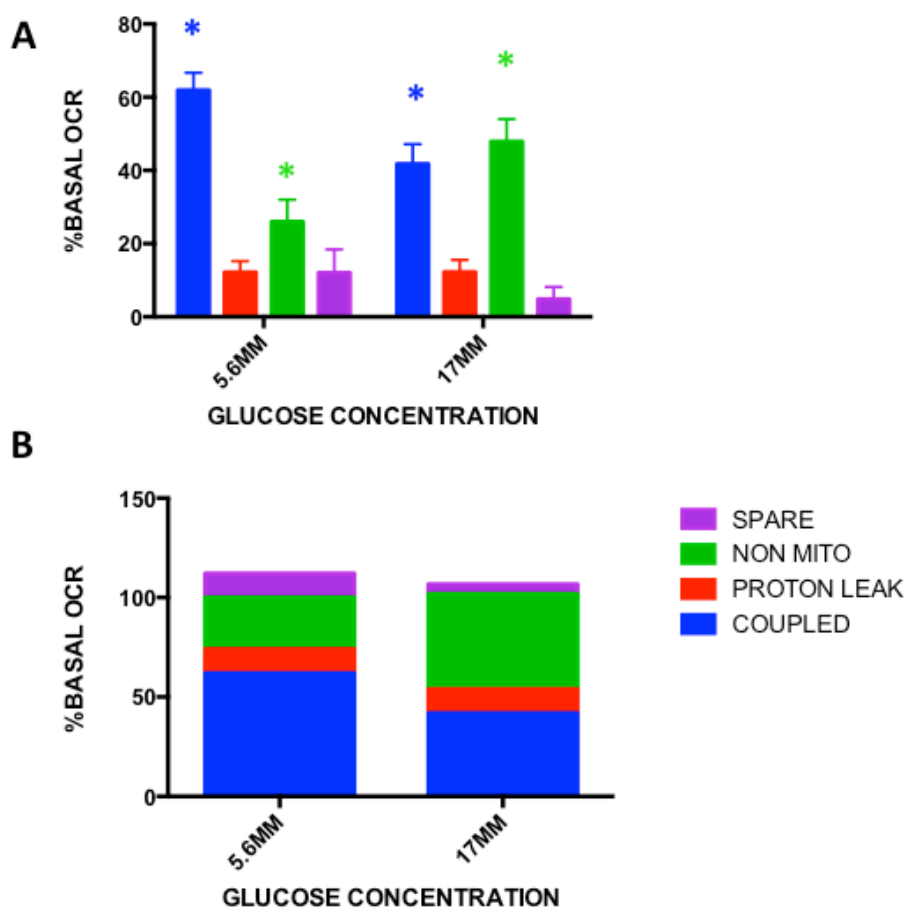


Figure 5.5: Effect of glucose concentration during IVM on mitochondrial function as determined by sequential addition of respiratory inhibitors. Oxygen consumption rate (OCR) coupled to ATP production (BLUE), OCR associated with proton leak (RED), non-mitochondrial OCR (GREEN) and spare respiratory capacity (PURPLE) are expressed as % basal OCR. Mean \pm SEM is presented in Panel A and mean presented in Panel B. Asterisks represent significant differences in that parameter between glucose concentrations (BLUE*= Coupled; $p = 0.01$ and GREEN*= Non-mitochondrial; $p = 0.02$)

5. 4.3 Experiment 5.3. Study 1: Effect of glucose concentration during IVM on resulting blastocyst gene expression.

Five blastocysts were analysed. Blastocysts C1-4 were from the 5.6-mM glucose IVM group and blastocyst H1 from the 17-mM glucose IVM group (Figures 5.7-5.11). Blastocyst C1 developed from an oocyte that had depleted all available glucose during IVM. Due to the small sample size and large biological variation, the effect of glucose concentration during IVM could not be evaluated and a descriptive analysis of the relative expression of each of the genes is therefore shown below both overall (Figure 5.6), and for each individual blastocyst (Figures 5.7-5.9).

Reference genes *SRP14* (Signal Recognition Particle 14) and *RPL32* (Ribosomal protein 32) (See Section 2.5.2) were further examined for suitability alongside the genes of interest using probe-based primers. *RPL32* showed higher expression than did *SRP14* in all samples (mean Ct 17.2, vs. 19.6 for *SRP14*) and was therefore used to calculate Δ Ct values. *GPX1*, *LDHA* and *POU5F1* showed the highest relative expression and were expressed in all blastocysts (Figure 5.6). *TFAM*, *SLC2A1*, *PFKM* and *CDX2* were not universally expressed and *CPT1A* and *NANOG* did not show expression in any of the five blastocysts. All blastocysts showed expression of at least six genes of interest in addition to the reference genes (Table 5.4).

	BLASTOCYST				
GENE	C1	C2	C3	C4	H1
<i>TFAM</i>	0.00921	0	0	0	0
<i>SLC2A1</i>	0	0	0	0	0.00010
<i>LDHA</i>	0.27042	0.08322	0.04966	0.21525	0.12729
<i>G6PD</i>	0.00027	0.00532	0.00002	0.00475	0.00281
<i>GPX1</i>	0.22463	1.39289	2.62175	0.24190	0.77179
<i>PFKM</i>	0.00233	0	0	0	0.00220
<i>CDX2</i>	0	0	0	0.00030	0
<i>TEAD4</i>	0.00039	0.00012	0.02053	0.00205	0.00677
<i>SOX2</i>	0.00185	0.00061	0.00139	0.00343	0.00337
<i>POU5F1</i>	0.25489	0.69544	1.60626	0.24622	0.88087

Table 5.4: Summary of relative expression (normalised to RPL32) of genes of interest in each of the five blastocysts. Blastocysts C1-4 are from COCs matured in control maturation medium (5.6 mM glucose) and blastocyst H1 from COC matured in high glucose medium (17 mM).

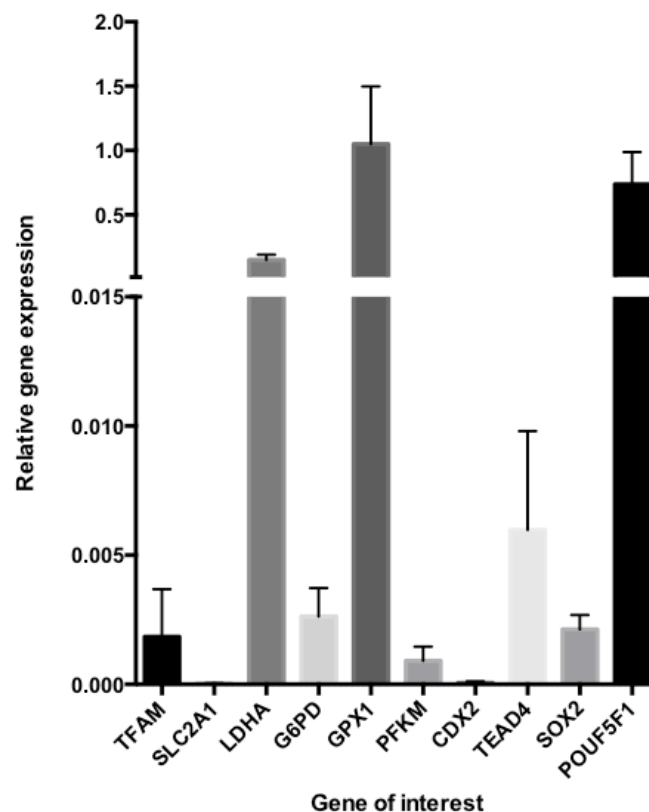


Figure 5.6: Relative expression (normalised to RPL32) of the ten target genes in the five blastocysts studied. Mean and SEM are depicted where appropriate. Only one embryo expressed SLC2A1 and CDX2 and a mean is therefore not presented.

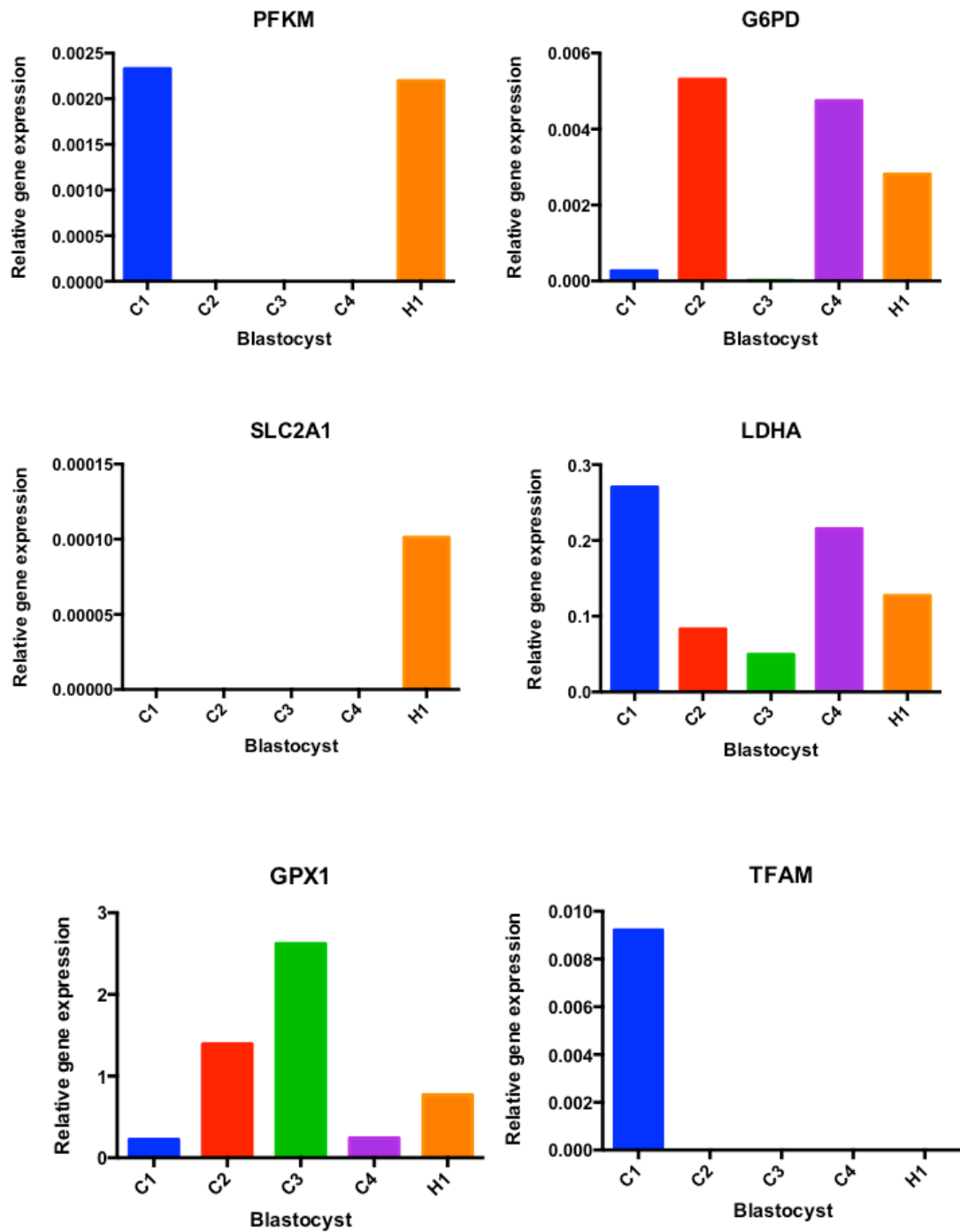


Figure 5.7: Relative expression levels (normalised to RPL32) of genes related to glucose metabolism and oxidative stress. Blastocysts C1-4 are from COCs matured in control maturation medium (5.6 mM glucose) and blastocyst H1 from COC matured in high glucose medium (17 mM). Empty bars denote lack of gene expression in that blastocyst.

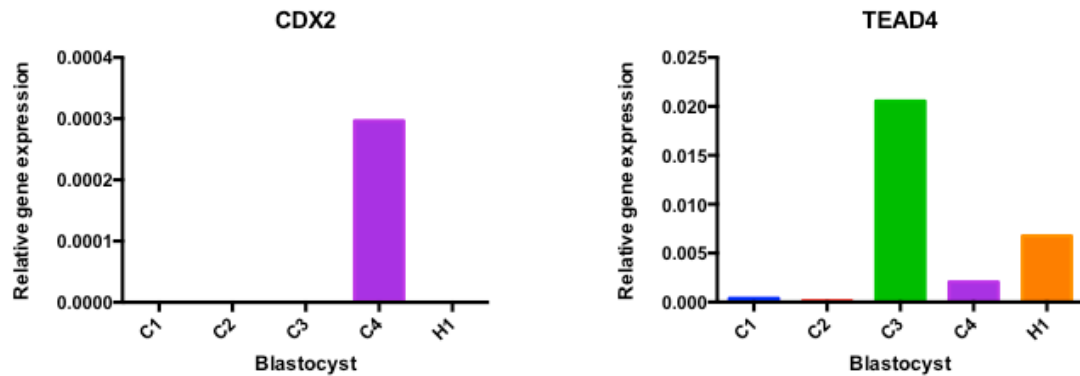


Figure 5.8: Relative expression levels (normalised to RPL32) of genes related to trophectoderm differentiation. Blastocysts C1-4 are from COCs matured in control maturation medium (5.6 mM glucose) and blastocyst H1 from COC matured in high glucose medium (17 mM). Empty bars denote lack of gene expression in that blastocyst.

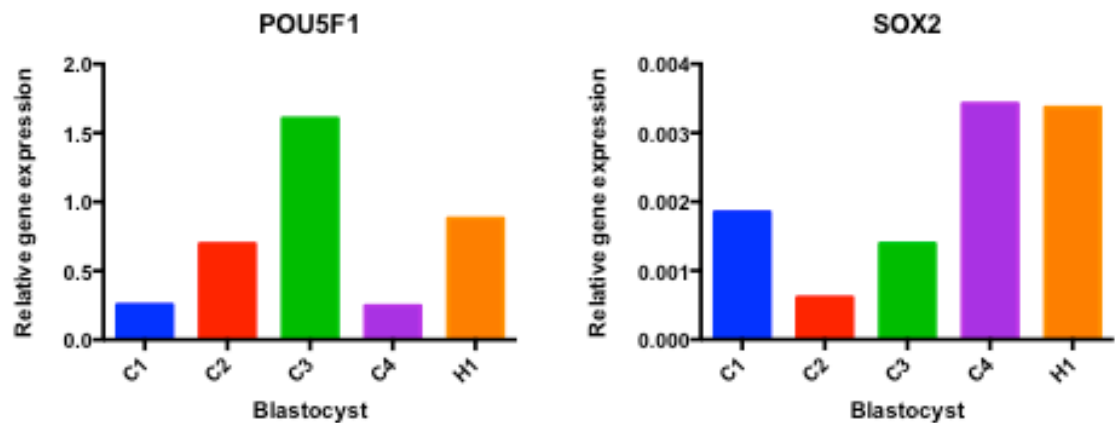


Figure 5.9: Relative expression levels (normalised to RPL32) of genes related to pluripotency. Blastocysts C1-4 are from COCs matured in control maturation medium (5.6 mM glucose) and blastocyst H1 from COC matured in high glucose medium (17 mM).

Study 2A- Pairwise comparison between relative expression of genes

Of the 90 pairwise comparisons performed (data not shown), five were either significant ($p < 0.05$) or approached significance ($p < 0.1$). *LDHA* and *GPX1* expression were negatively correlated ($r^2 = 0.8$; $p = 0.04$; Figure 5.10A) and both were correlated to *POU5F1* expression ($r^2 = 0.75$; $p = 0.06$; Figure 5.12D and $r^2 = 0.9$; $p = 0.02$; Figure 5.10B respectively). *GPX1* expression was also correlated to *TEAD4* expression ($r^2 = 0.7$; $p = 0.08$; Figure 5.10C) and *TEAD4* expression was correlated to *POU5F1* expression ($r^2 = 0.9$; $p = 0.03$; Figure 5.10E).

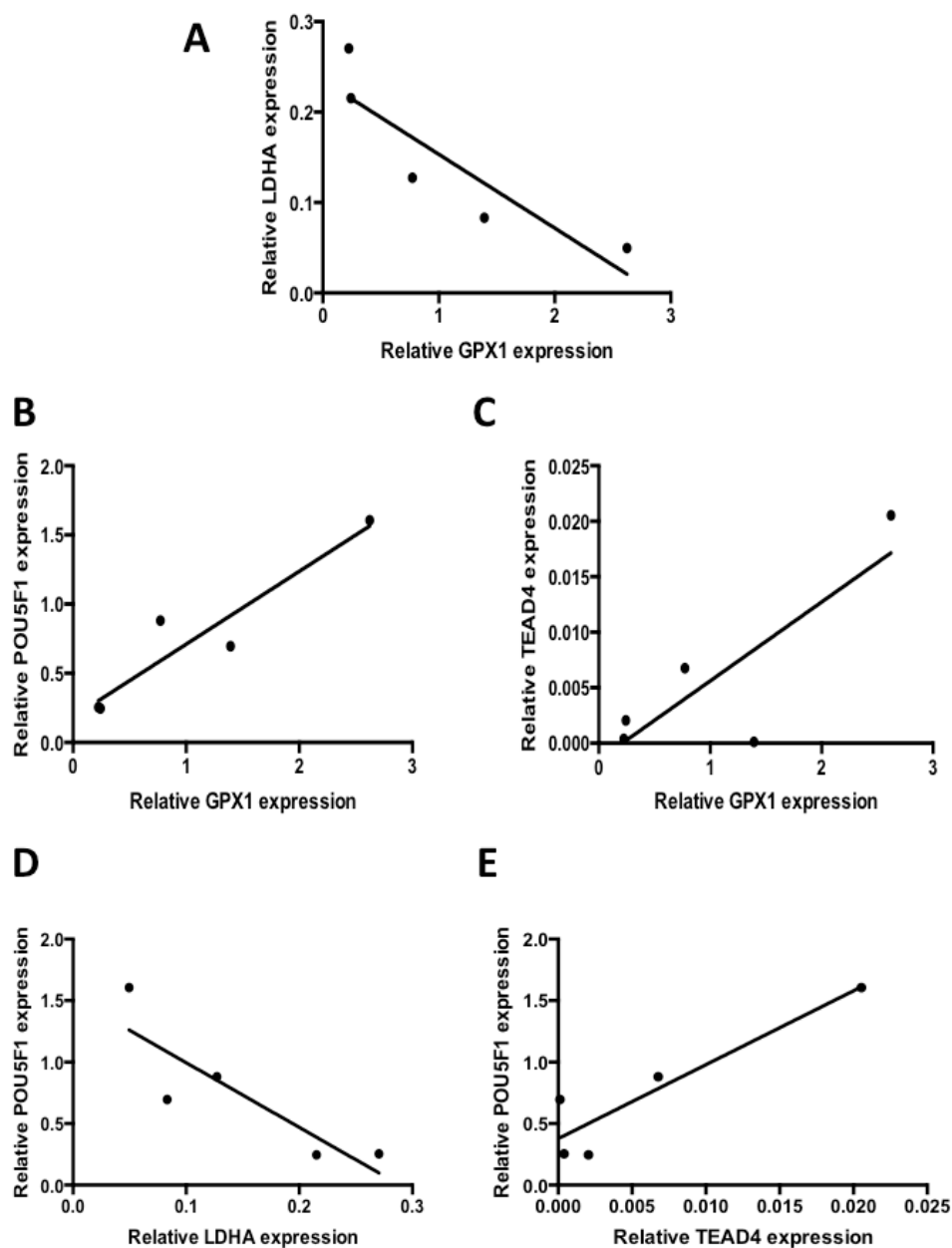


Figure 5.10: Correlation of relative gene expression levels in individual blastocysts. A: GPX1 and LDHA ($r^2 = 0.8$; $p = 0.04$); B: GPX1 and POU5F1 ($r^2 = 0.9$; $p = 0.02$); C: GPX1 and TEAD4 ($r^2 = 0.7$; $p = 0.08$); D: LDHA and POU5F1 ($r^2 = 0.75$; $p = 0.06$); E: TEAD4 and POU5F1 ($r^2 = 0.9$; $p = 0.03$).

Study 2B- Correlation between relative gene expression and corresponding IVM metabolic parameters.

Glucose consumption, lactate and pyruvate production rates and lactate : glucose ratios during IVM are shown below for each of the COCs from which the five blastocysts developed (Figure 5.11; Pyruvate was only measurable in 3/5 due to technical issues). Blastocyst C1 developed from an oocyte that depleted all available glucose during the 30-hour maturation period.

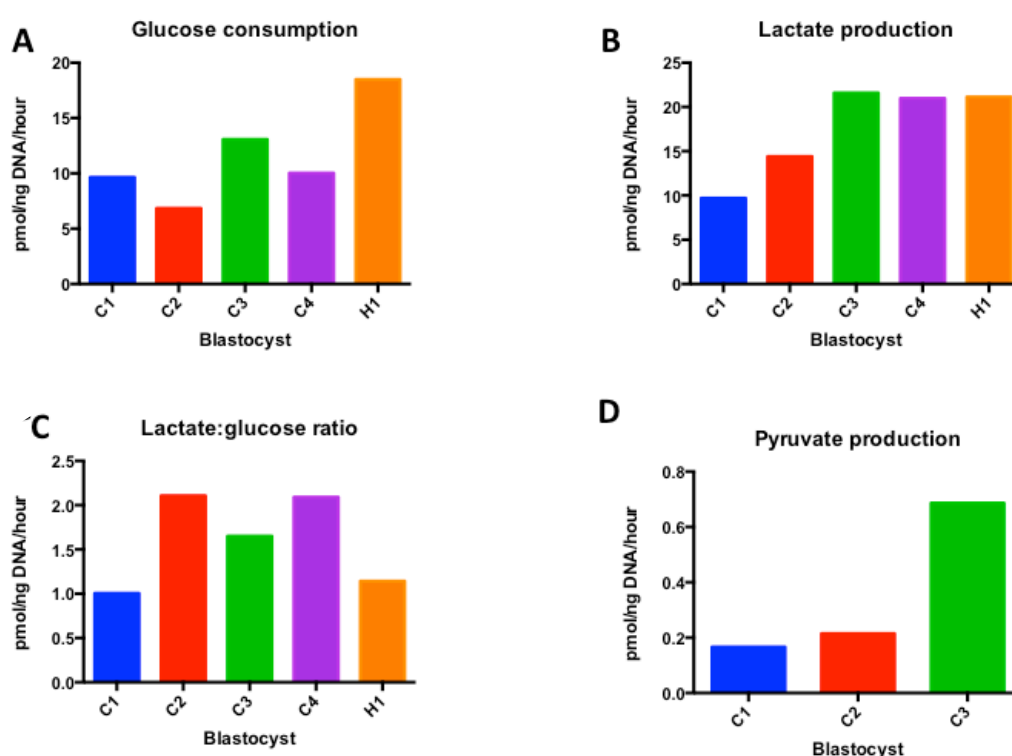


Figure 5.11: Glucose consumption (A), lactate production (B), lactate: glucose ratio (C) and pyruvate production (D) in each of the five blastocysts. All bars represent the mean of a technical triplicate (CV<10%) in pmol/ ng DNA/ hour. Blastocysts C1-4 were from COCs matured in control maturation medium (5.6 mM glucose) and blastocyst H1 from COC matured in high glucose medium (17 mM).

The association between glucose consumption rate, lactate production rate and lactate : glucose ratio during IVM and relative expression of each of the ten genes in the resulting blastocyst was explored (data not shown). Associations were either significant ($p < 0.05$) or approached significance ($p < 0.1$) for two comparisons however were driven by a single blastocyst. Glucose consumption rate was positively correlated to *SLC2A1* expression (the only blastocyst with *SLC2A1* expression [H1] developed from the COC with the highest glucose consumption; $r^2 = 0.8$; $p = 0.06$). Lactate production rate was negatively correlated to *TFAM* expression (the only blastocyst with *TFAM* expression [C1] developed from the COC with the lowest lactate production; $r^2 = 0.7$; $p = 0.08$)

Study 2C- Correlation between relative gene expression and corresponding morphokinetics during development

Time of blastocyst formation and blastocyst diameter at blastulation are shown below for each embryo (Figure 5.12A and B). The association between relative gene expression in the blastocyst and time of blastulation and diameter was found to be either significant ($p < 0.05$) or approaching significance ($p < 0.2$) in three of the 20 comparisons. Blastulation time was negatively correlated to *POU5F1* ($r^2 = 0.6$; $p = 0.17$; Figure 5.12C) and positively correlated to *CDX2* expression ($r^2 = 0.9$; $p = 0.02$) and diameter at blastulation was positively correlated to *TEAD4* expression ($r^2 = 0.6$; $p = 0.1$). The latter two associations were however driven by a single blastocyst.

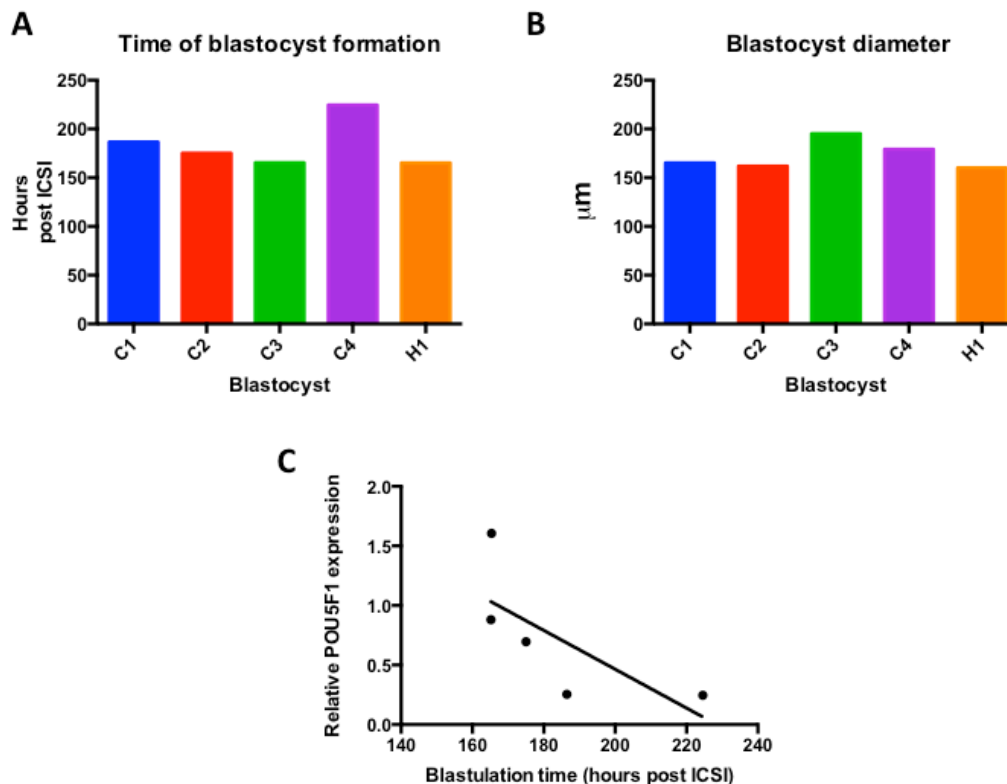


Figure 5.12: Time of blastulation (A) and diameter at time of lysis (B) for each of the five blastocysts. Blastocysts C1-4 are from COCs matured in control maturation medium (5.6 mM glucose) and blastocyst H1 from COC matured in high glucose medium (17 mM). Panel C: Correlation of blastulation time and relative *POU5F1* expression in the blastocysts C; $r^2 = 0.6$; $p = 0.17$).

5.5 Discussion

The effect of hyperglycaemia during IVM on oocytes, COCs and embryos of other species is well described (reviewed by Frank et al., 2014), however this is the first report to investigate the effect of glucose concentration during the period of IVM on equine COCs and resulting blastocysts. Glucose concentration during IVM (5.6 vs. 17 mM) did not influence developmental parameters, morphokinetics of early embryo development, glucose consumption, lactate/ pyruvate production or basal OCR. However, IVM at 17 mM glucose resulted in changes in mitochondrial function: decreased ATP-coupled respiration and increased non-mitochondrial respiration. Regrettably, blastocyst development rates were low and the effect of IVM glucose concentration on blastocyst gene expression and morphokinetics could therefore not be determined. However, the expression of glucose metabolism genes *SLC2A1*, *LDHA*, *G6PD*, *PFKM* and the TE marker *TEAD4* is reported for the first time in equine blastocysts and further description of *POU5F1*, *SOX2* and *CDX2* adds to our limited knowledge of dynamics of cell lineage-associated gene expression in the horse.

Hyperglycaemia was not associated with a change in measured markers of COC carbohydrate metabolism

Studies in both mice and cattle have demonstrated a reduction in glucose uptake by COCs and embryos in hyperglycaemic conditions attributed to a downregulation of glucose transporters (Frank et al., 2014; Sutton-McDowall et al., 2010; Wang et al., 2012, 2010). In contrast, variation in the medium glucose concentration had no effect on glucose consumption in the present study. The degree of COC and embryo glucose tolerance appears to be species specific, although the mechanism is unknown. While hyperglycaemia (~ 5 mM) in early development dramatically lowers embryo development in the mouse, hamster, sheep and cow (Chatot et al., 1989; Furnus et al., 1997; Seshagiri and Bavister, 1989; Thompson et al., 1992), porcine and equine embryos develop normally (Choi et al., 2015; Sturmey and Leese, 2003). In fact, equine embryos tolerate glucose concentrations up to 20 mM during this time with no apparent effect on developmental potential (Choi et al.,

2015). In pig embryos, in which lipids are abundant as is assumed for the equine oocyte, it has been proposed that high glucose levels are protective in that they prevent the preferential oxidation of intracellular fatty acids and hence potentially prevent excess ROS production (Sturmey and Leese, 2003).

Glucose transporters (GLUT1-12) are differentially expressed in different tissues and also vary in substrate affinity (Zhao and Keating, 2007). GLUT1 is a high affinity glucose transporter ($K_m = 3-7$ mM) shown to be present in cumulus cells and be downregulated in the face of hyperglycaemia (Wang et al., 2010). In this regard it is interesting to consider another equine tissue in which GLUT1 activity has been evaluated. A common clinical problem in equine medicine is laminitis, in which the digital lamellae (structures that attach the distal phalanx to the hoof wall) separate from the hoof capsule and lead to extreme pain and lameness, resulting in morbidity and even necessitating eventual euthanasia. Laminitis is closely associated with obesity, metabolic syndrome and insulin resistance; however the mechanism by which these changes induce the pathology in the hoof is unknown. One theory put forward was that the digital lamellae in the hoof were starved of glucose due to impaired GLUT4 mobilisation, which typically accompanies insulin resistance. However, a study examining the *GLUT1* and *GLUT4* (*SLC2A1* and *4*) expression in equine lamellar explants discounted that theory, as glucose transport in the hoof was found to be mediated by only the insulin insensitive GLUT1 receptor (Asplin et al., 2011). Moreover, in the context to the present study, they showed that glucose uptake by the lamellar explants increased up to a concentration of 15 mmol/L and did not fall as a result of *GLUT1* downregulation until 21 mmol/L. In contrast, a study examining glucose uptake in human keratinocytes demonstrated a decrease in glucose uptake at 4 mM glucose (Spravchikov et al., 2001). While there is variation in glucose transport properties between tissues, the study by Asplin lends weight to the theory that higher concentrations of glucose than used in the present study may be required in the equine COC/embryo to cause a decrease in glucose uptake.

A limitation of the current experimental method, which measured disappearance of glucose and appearance of lactate, is that it provides only a global estimate of glycolysis through the glycolytic index (lactate : glucose ratio). Glucose can additionally be metabolised via the pentose phosphate pathway (PPP), Poly- O-L pathway and HBP or directly oxidised via OXPHOS (Figure 5.13). The change in proportional flux through these individual pathways is perhaps more informative than the overall consumption. The HBP in particular has received attention in recent years as a potential central mediator of glucotoxicity in COCs, embryos and somatic cells (Pantaleon, 2015; Rolo and Palmeira, 2006; Sutton-McDowall et al., 2010; Wong et al., 2015). In somatic cells under normoglycaemic conditions, the HBP accounts for 1-3% of glucose metabolised (Marshall et al., 1991). However in the maturing COC, it has a particularly central role: hyaluronic acid, a component essential for cumulus expansion, is an end product of the HBP and up to 25% of glucose taken up by the COC is metabolised via the HBP at the end of IVM in the cow (Sutton-McDowall et al., 2004). Another fate of the end product of the HBP, UDP-N-Acetylglucosamine, is β -O linked glycosylation of proteins (O- GlcNAcylation; Figure 5.13). O-GlcNAcylation acts in a similar way to phosphorylation to regulate protein function (Marshall et al., 1991) and as such, a certain level is required for optimal cell function but excess may be detrimental. Hyperglycaemia has been shown to increase flux through the HBP thereby increasing O- GlcNAcylation, which in the COC, may have profound downstream negative effects on blastocyst development (Pantaleon et al., 2010; Sutton-McDowall et al., 2006; Wong et al., 2015). In murine and bovine studies, embryotoxic effects of hyperglycaemia are ameliorated if the O- GlcNAcylation is inhibited, adding further evidence to the proposition that increased HBP activity is a major cause of glucotoxicity (Pantaleon et al., 2010; Sutton-McDowall et al., 2006). Interestingly, aberrant O- GlcNAcylation of reproductive tissues has also been implicated as a cause of negative pregnancy and birth outcomes secondary to hyperglycaemia (Brown et al., 2018).

In addition to increased HBP flux, hyperglycaemia causes an increase in conversion of glucose to sorbitol (Poly-O-L pathway), and both decrease PPP activity through negative feedback on Glucose-6-phosphate dehydrogenase (G6PDH, Figure 5.13) (Colton et al., 2003; Rolo and Palmeira, 2006; Sutton-Mcdowall et al., 2006; Wong et al., 2015). As the PPP generates NADPH, it contributes to redox balance by ensuring the NADPH: NADP ratio is maintained in a reduced state. A high NADPH: NADP is essential for optimal ROS scavenging via enzymes such as the glutathione reductases.

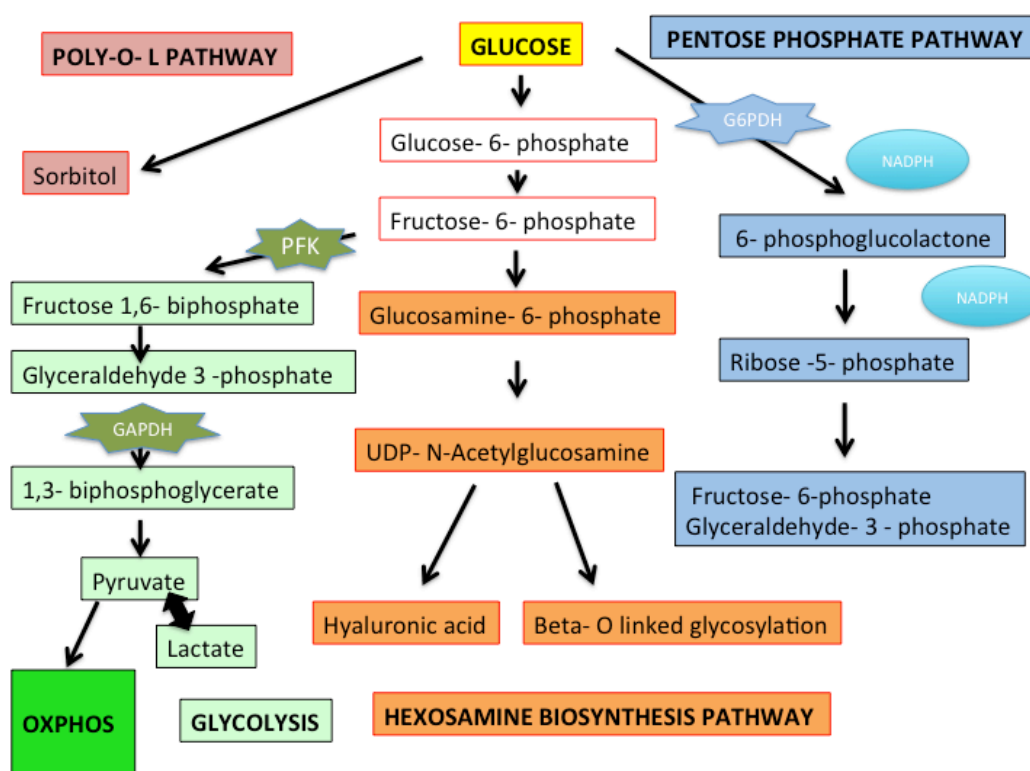


Figure 5.13 A simplified schematic showing the potential routes of glucose metabolism. Enzymes and steps relevant to this discussion are highlighted. Abbreviations: PFK: Phosphofructokinase, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, G6PDH: Glucose-6-phosphate dehydrogenase, OXPHOS; Oxidative phosphorylation. Adapted from (Sutton et al., 2003).

Hyperglycaemia has also been shown to reduce the proportion of glucose that is directly oxidised, as it lowers pyruvate dehydrogenase activity (PDH). PDH converts pyruvate to acetyl-CoA and as such, low activity means that pyruvate is not oxidised but is preferentially converted to lactate. This is a well-recognised phenomenon in diabetic cardiomyopathy (DCM) in which resultant overutilisation of fatty acids for oxidation (as a source of ATP in the absence of pyruvate availability) is linked to many of the negative effects of DCM. Thus, PDH promoters are currently of interest as potential drug targets for amelioration of DCM-related pathology (Page et al., 2015). In the current study, there were no differences in glycolytic index associated with 17 mM vs. 5.6 mM glucose; however it would be interesting to re-evaluate this at higher glucose concentrations, and to concurrently evaluate PDH expression.

Hyperglycaemia resulted in mitochondrial dysfunction

In the current study, there was a decrease in ATP-coupled OCR at 17 mM glucose during IVM, compared to 5.6 mM, associated with an increase in the proportion of non-mitochondrial respiration (OCR not associated with the electron transport chain). Both these measures suggest compromised mitochondria and an increase in ROS production in COCs matured in hyperglycaemic conditions. This finding is in agreement with studies in both oocytes and somatic cells exposed to high glucose levels (Hashimoto et al., 2000; Rolo and Palmeira, 2006; Wang et al., 2010). Recent studies using the Seahorse XF analyser also demonstrated a decrease in ATP-coupled OCR in response to hyperglycaemia in both renal mesangial and tubular cells (Czajka and Malik, 2016) and peripheral mononuclear cells (Hartman et al., 2014). A decrease in coupling has also been demonstrated in myocardium, attributed to overutilisation of fatty acids for oxidation and subsequent upregulation of uncoupling proteins (UPCs) (Rider et al., 2012). Interestingly, consistently lower ATP levels are reported in oocytes and cumulus cells as a sequela to hyperglycaemia (Chang and Moley, 2004; Colton et al., 2003; Wang et al., 2012, 2010). While not directly measured in the current study, it could be extrapolated that as the basal OCR did not differ significantly between conditions and the ATP linked proportion was significantly decreased at 17 mM, this would also result in decreased ATP production.

According to Rolo and Palmeira, (2006) the consequences of hyperglycaemia in somatic cells can be traced back to an increase in ROS production and impaired ROS scavenging. One of the principal factors determining ROS production is the redox state of the mitochondrial membrane and in turn, mitochondrial efficiency is central to regulating this response. It has been elegantly demonstrated by a series of experiments from Kelle Molley's laboratory that mitochondria both in the oocyte and cumulus cell are a target for hyperglycaemia (Chi et al., 2000; Ratchford et al., 2007; Wang et al., 2012, 2010, 2009). Using the streptozotocin (STZ) injected mouse model to create diabetes through destruction of pancreatic β cells, they have shown that oocytes from those mice have increased numbers of fragmented mitochondria, a more aggregated mitochondrial pattern and morphological changes in remaining individual mitochondria. All these findings combine to decrease mitochondrial efficiency. Further inefficiencies stem from aberrations in glucose pathways mentioned above. Firstly, decreased PPP activity leads to decreased NADPH levels and a subsequent increase in the electron donor NADH. This results in an elevated mitochondrial membrane potential due to increased electron transfer chain activity, and also in increased ATP/ADP ratio. Consequently, there is negative feedback on Complex III, due to the high electrochemical gradient, and subsequently a resultant accumulation of electrons within the chain which leads to partial reduction of oxygen ($O_2 + e^- \rightarrow \cdot O_2^-$) and thus increased super oxide (SO) formation (Rolo and Palmeira, 2006). This mechanism has been confirmed in bovine aortic endothelium exposed to hyperglycaemia whereby ROS production is lowered in response to treatment with FCCP, which lowers membrane potential by allowing free passage of protons out of the intermembrane space (Nishikawa et al., 2000). An increase in ROS in response to hyperglycaemia (20mM) has also been demonstrated in bovine oocytes Hashimoto et al., (2000). Excess superoxide can inhibit the glycolytic enzyme Glyceraldehyde-3 phosphate dehydrogenase (GAPDH, Figure 5.13), which prevents completion of glycolysis and thus the excess glucose in hyperglycaemia is shunted through the HBP (Rolo and Palmeira, 2006). Consistent with these findings, addition of antioxidants improves bovine IVF outcome in the face of hyperglycaemia during embryo culture (Iwata et al., 1998).

Hyperglycaemia had no effect on developmental parameters or morphokinetics

Much of the research investigating the effects of hyperglycaemia on oocytes, COCs and embryos has utilised the Akita mouse model which has a mutation in the insulin-2 gene (*Ins2*) leading to insulin-dependent diabetes (Wang et al., 1999). Using this mouse model, it has been demonstrated that oocytes from diabetic mice exhibit slower progression to MII in response to gonadotropins, and also show meiotic spindle defects (Chang et al., 2005). These authors have attributed the delay in meiotic progression to loss of oocyte-cumulus cell communication as a result of two separate processes; increased extrinsic apoptosis and decreased connexin expression. The connexin proteins are vital for gap junction formation between the oocyte and cumulus cells and progression to Metaphase II is reliant in particular on connexin 43 (Ackert et al., 2001). Ratchford et al., (2008) demonstrated that there was a 60% decrease in cumulus cell connexin expression in response to hyperglycaemia in the Akita mouse. In the present study, there was no difference in maturation rate associated with hyperglycaemic conditions, however meiotic status was evaluated at 30 hours only, so it is unknown whether the kinetics differed between the two groups.

The assessment of morphokinetics is an emerging field, especially in human ART, and most research to date has concentrated on developing algorithms for selection of high quality embryos (meta-analysis by Chen et al., 2017). Notably, there are no data on the effect of glucose concentration on embryo morphokinetics. Oocytes from the diabetic Akita mouse model are reportedly smaller than those from normoglycaemic mice (Chang et al., 2005). Not unexpectedly, unlike prolonged exposure during folliculogenesis *in vivo*, short exposure to increased glucose during IVM in the present study did not influence oocyte size. With higher numbers and a system with higher blastocyst development rates, it would be interesting to evaluate the kinetics of blastocyst development, since there is evidence to suggest retardation in murine embryo development in response to hyperglycaemia. When murine embryos were exposed to increased glucose concentrations (25.56 mM) at

the 2-cell stage, decreased hatching and lower blastocyst cell numbers were observed on Day 5 (Fraser et al., 2007).

Hyperglycaemia and blastocyst gene expression

Blastocyst rates were too low in this study to satisfy the original experimental aim of identifying an effect of glucose concentration on developmental potential or blastocyst gene expression. Large animal studies performed in buffalo and cattle have demonstrated a consistent decrease in blastocyst rate attributable to high glucose during IVM (10 and 20 mM, compared to 5.6 mM) (Hashimoto et al., 2000; Kumar et al., 2012). While DMEM/F12 (17 mM glucose) has been successfully used for IVM in the horse (blastocyst rates up to 17%; Galli et al., 2016) the effect of glucose has not been independently evaluated. In the current study, due to the low overall rate of blastocyst development, differences in blastocyst development could not be tested. However, it is interesting to comment on the relative expression of the genes measured in this study since most have not previously been described in the equine blastocyst. There was universal expression of *GPX1*, *LDHA* and *G6PD*. Only one blastocyst (the 5.6 mM COC that exhausted glucose during IVM) expressed *TFAM* and one (17 mM group) expressed *SLC2A1*. Two expressed *PFKM* and none *CPT1A*. Carnitine palmitoyl transferase 1 (CPT1) is the enzyme responsible for the transfer of fatty acids across the outer mitochondrial membrane and is the rate-limiting step of β -oxidation. CPT1 has three isoforms 1A, 1B and 1C. While *CPT1A* has been detected in bovine blastocysts, it is possible that one of the other two isoforms is of more importance in the equine blastocyst. In two bovine studies investigating the effect of varying IVM conditions (the first investigating oxygen tension [5%/20%] and the second, glucose concentration in the face of lipolysis-like conditions) on blastocyst gene expression, no differences were identified in gene expression attributable to IVM condition (Bermejo-Álvarez et al., 2010 and De Bie et al., 2017 respectively) however, there was universal expression of *TFAM*, *CPT1A* and *SLC2A1*.

Expression of *TFAM*, which codes for a key activator of mitochondrial transcription, has been described in equine blastocysts in two studies from the same laboratory (Hendriks et al., 2015, 2018). They first examined *TFAM* and other genes related to mitochondrial replication between *in vivo*-derived blastocysts from young and old mares and IVP blastocysts and reported no differences in *TFAM* expression. In that study, only *GPX3*, a glutathione peroxidase, was differentially expressed; namely, lower expression in IVP compared to *in vivo* derived blastocysts from young mares. In the 2018 study, the temporal expression of *TFAM* was evaluated in relation to re-initiation of mtDNA replication. The increase in mtDNA at the blastocyst stage was preceded by an increase in *TFAM* expression on Day 4 after ICSI. It is not clear why only one blastocyst in the present study expressed *TFAM* and one the glucose transporter gene *SLC2A1*. However, it is noteworthy that Blastocyst H1, the only embryo to express *SLC2A1*, was the only blastocyst to develop from a COC in the 17-mM IVM group.

Information on the temporal dynamics and regulation of cell lineage allocation in the equine embryo is limited. Choi et al. first described *POU5F1*, *SOX2*, *NANOG* and *CDX2* in equine blastocysts in 2009 and showed that there was indiscriminate presence of POU5F1 protein across the entire IVP blastocyst (Day 7-11) whereas it was confined to the ICM in *in vivo* derived blastocysts (Day 7-10). They then showed that TE levels of POU5F1 fell when the IVP embryos were transferred to the uteri of mares for 2 to 3 days (IVP-ET). To confirm these immunohistochemistry findings, isolated TE and ICM cells from *IVP-ET* blastocysts were evaluated for mRNA expression, which revealed higher expression of pluripotency markers *POU5F1*, *SOX2* and *NANOG* in the ICM compared to the TE cells, verifying the immunohistochemistry results. A second study, published as a short communication, compared the overall expression of *POU5F1*, *NANOG*, *SOX2*, *GATA6* and *CDX2* between *in vivo* and IVP embryos of different developmental stages (morula, early blastocyst and expanded blastocyst) and found that in *in vivo* derived embryos *POU5F1*, *NANOG* and *SOX2* expression decreased between the early blastocyst and blastocyst stage and *CDX2* increased between morula and expanded blastocyst (Paris et al., 2012). In contrast, they did not detect *CDX2* mRNA at any

stage in IVP embryos and reported a decrease in *POU5F1* expression overall (Paris et al., 2012). A third study, by Iqbal et al., (2014), reported next generation sequencing data of isolated ICM and TE cells from *in vivo* derived Day-8 equine blastocysts. These authors also found *POU5F1* to be expressed in both the ICM and TE, but reported a two to four fold increase in the ICM. Human and bovine embryos also display delayed loss of *POU5F1* in the TE compared to the mouse (Berg et al., 2011; Niakan and Eggan, 2013).

In the present study, the five blastocysts had high universal expression of *POU5F1*, perhaps the most widely studied developmental marker given its important role in regulating and maintaining pluripotency. With increased interest in embryonic stem (ES) cells for clinical use in horses, several studies have reported *POU5F1* expression in isolated equine ICM cells and ICM outgrowths after several passages to demonstrate pluripotency (Desmarais et al., 2011; Guest and Allen, 2007). *POU5F1* protein was detected in all cells in equine IVP blastocysts in two separate studies (Choi et al., 2015; Choi et al., 2009). In the present study there was also universal expression of *SOX2* while significantly, none of the five blastocysts expressed *NANOG*. There is known species variation in *NANOG* expression and its temporal role in epiblast formation. For example it is not detected in the pig embryo (Kuijk et al., 2008) while in the cat it is only detectable at low levels (Filliers et al., 2012). Heterogeneity also exists within the same species as reported by Kimber et al., (2008) who found non-universal expression of *SOX2*, *NANOG* and *POU5F1* in human blastocysts (5/13, 12/13 and 12/13 respectively). When equine IVP embryo culture systems were being developed, a high incidence of an-embryonic, trophoblast-only pregnancies after transfer was reported (Hinrichs et al., 2007). Given the role of *NANOG* in establishing the epiblast which later gives rise the embryo proper, it would have been interesting to know the *NANOG* mRNA status of those failed pregnancies. As it was detected in all three equine embryo studies to date, the reason for the lack of expression in the five blastocysts in the current study is unknown. It is unlikely to be a primer fault as it was detected in cumulus cells (Chapter 6) but could be due to differences in culture conditions, embryo stage or possibly individual animal variation.

Considering the TE marker genes, only one embryo expressed the trophoblast-specific transcription factor *CDX2* while expression of *TEAD4*, a transcription factor upstream of *CDX2* (Yagi et al., 2007) was universal but at varying levels. Choi et al. (2009) found high *CDX2* expression in IVP-ET blastocysts, but did not evaluate IVP blastocysts. Paris et al., (2012) failed to detect *CDX2* in equine IVP blastocysts while in a separate study they reported a clear increase in mRNA expression between the morula and blastocyst stage in *in vivo* derived equine embryos in line with an increase in number of TE cells (Paris et al., 2011). Given this strong temporal relationship in expression, it is worthwhile noting that all blastocysts in the present study were lysed within a similar time frame (25.9 ± 1.7 hours post start of blastulation), and as such it is possible that all the blastocysts were lysed before sufficient TE cells were present and hence detectable amounts of *CDX2* mRNA. As *TEAD4* acts upstream of *CDX2*, the presence of *TEAD4* in all blastocysts supports this idea. Notably, expression of *CDX2* protein was found universally in IVP equine blastocysts cultured for 24 hours after recognition of blastulation, similar to the current study (Choi et al., 2015). It was interesting that the single blastocyst expressing *CDX2* (Blastocyst C4) also had the lowest *POU5F1* expression, suggesting a reciprocal relationship between these transcription factors, in agreement with the literature in other species (Filliers et al., 2012; Kuijk et al., 2008; Strumpf et al., 2005).

Due to the low number of blastocysts obtained, the effect of glucose during IVM on the expression of pluripotency or TE marker genes in the equine embryo could not be determined. In other species, hyperglycaemia affects cell lineage allocation. In both the mouse and rat, *in vivo* diabetic models have generated data demonstrating that blastocysts recovered from diabetic mice have a decreased number of ICM cells resulting in a decreased ICM :TE ratio (Lea et al., 1996; Leunda- Casi et al., 2002; Pampfer et al., 1990). The mechanism behind this decreased ICM :TE ratio is likely mediated by increased apoptosis via upregulation of BAX leading to increased DNA fragmentation (Chi et al., 2000; Moley et al., 1998), however the reason for the increased sensitivity of the ICM cells is unclear. There is one study on equine embryos by Choi et al., (2015) performed during early embryo development *in vitro*

(Days 0-7 after ICSI) which also demonstrated that the medium glucose concentration during Days 0-5 of culture influenced cell allocation. These authors reported that while the blastocyst rate remained constant, there was a decrease in total cell number and presumptive epiblast cell number when injected oocytes were cultured at 5 mM glucose compared to 0 mM. While expression of pluripotency genes was not measured in any of the above studies it could be postulated that those embryos with decreased number of ICM cells would also have decreased *POU5F1* expression given the preferential ICM expression in the mouse and rat. It would be interesting to discover if the same was true for the horse given the lack of *POU5F1* localisation to the ICM.

While the ambitious aim of identifying associations between blastocyst gene expression, COC metabolism and morphokinetics of embryo development could not be addressed due to low embryo numbers, this study served as proof of principle of the value of this experimental design. It is possible to speculate on some interesting observations. Blastocyst C1 developed from a COC that depleted all available glucose during IVM, and of the five embryos, it had the highest relative expression of PFKM and LDHA, was the only embryo to express TFAM and had the lowest GPX1 expression. It is possible that high rates of COC glucose metabolism programmed a high rate of glycolysis in the blastocyst as demonstrated by high PFKM and LDHA expression. The effect of the complete depletion of glucose during IVM could also not be investigated, however the low expression of *GPX1*, is of interest given its association with developmental potential in other species. *GPX1* has been shown to be upregulated both in higher quality bovine embryos (Cebrian-Serrano et al., 2013) and in porcine embryos with enhanced survival after cryopreservation and thawing (Castillo-martín et al., 2014). An equine study also reported a decrease in *GPX3* expression in IVP compared to *in vivo* derived blastocysts, further pointing to the potential association of glutathione peroxidase expression with developmental competence. In line with *GPX1* potentially being a marker for blastocyst quality, it is interesting that Blastocyst C3, which had the highest *GPX1* expression, had the highest *POU5F1* expression in addition to being the largest and fastest to develop. *POU5F1* expression was also positively correlated to the time of blastocyst

development overall. Equine clinical ICSI data have recently shown that likelihood of foal production is higher for blastocysts that form on Day 7 (52%) than those that form on Day 8 (38%) (Claes et al., 2018). The slowest embryo to develop (Blastocyst C4) began blastulating at 224 hours (Day 9) and had the lowest POU5F1 expression and very low GPX1 expression, further suggesting that POU5F1 and GPX1 are potential developmental markers. It was also notable that this embryo was the only one to express CDX2.

Conclusions

The results of this study demonstrate that a change in metabolism occurs at the level of the mitochondria when equine COCs undergo IVM in the presence of 17 mM vs. 5.6 mM glucose, however further work is needed to unravel the differences in pathway allocation and their consequences, if any, to developmental potential. The role of the HBP and direct measurement of ROS would be particularly interesting areas for future research in the horse. Currently, it appears that the equine COC can adapt to differing glucose concentrations, however it is not established whether the imbalance of glucose pathways and altered mitochondrial function have negative downstream effects on pregnancy outcome or the offspring.

The novel experimental design and blastocyst gene expression data represent exciting avenues for future study, in both the horse and other species.

Chapter 6: The use of transcript profiling of single COCs to understand the impact of glucose concentration during IVM.

6.1 Introduction

Interpretation of data in Chapter 5 indicated that increased glucose concentration during IVM resulted in altered mitochondrial function in the COC. However, no effect on gene expression in the blastocyst was observed. In the experiments reported in this chapter, the immediate effects of hyperglycaemia on the cumulus cell and oocyte transcriptome were examined in an attempt to identify the mechanisms and consequences of the observed alteration in mitochondrial function. However, this approach presented challenges in interpretation and as such a brief literature review on aspects of transcriptomic analysis specific to this chapter is given below.

Interpreting the oocyte transcriptome given the dynamic nature of maternal mRNA

While it is accepted that the majority of mRNA within the mature oocyte is synthesized in the growth phase, continuing up to the point of chromatin condensation, there is controversy (a) on whether or not transcription occurs during meiotic resumption (i.e. final maturation of the oocyte) and (b) the interpretation of differentially expressed genes in oocytes between different conditions.

Maternal mRNA is produced in the nucleus and exported to the cytoplasm during the growth phase of the oocyte. While in most cells mRNA has a very short lifespan, the oocyte is unique in being able to store cytoplasmic mRNA in a stable state by shortening the polyA tail (20-40 bp). The subsequent translation of mRNA is then regulated by temporal polyadenylation (lengthening to 80-250 bp) or deadenylation (Rodriguez and Farin, 2004) and timely availability of specific mRNAs is crucial for meiotic resumption, fertilization and early cleavage divisions through to embryonic genome activation [EGA] (Mamo et al., 2011). Polyadenylation involves the phosphorylation of CPE (cytoplasmic polyadenylation element)-binding proteins

(CPEs are uridine-rich sequences at the 3'UTR). In *Xenopus* oocytes, in the stable (inactive) state, these proteins are associated via a closed-loop structure with maskin proteins at the 5' end, however when phosphorylated they are no longer associated and other proteins such as Poly-A binding proteins act to elongate the PolyA tail (Lin, 2010). This mechanism is fairly well conserved in mammalian species (Reyes and Ross, 2016).

The mechanisms by which temporal translation of maternal mRNAs is controlled are still unclear. Recently Cakmak *et al.*, (2016), using IL-7 as an example (an oocyte specific cytokine, secretion of which increases during final oocyte maturation), showed that translation in the oocyte was promoted by the presence of cumulus cells. They measured polysome-associated mRNA, an indicator of translation, in response to AREG (an epidermal-like growth factor) in cumulus-surrounded oocytes. In comparison to denuded oocytes, they reported increased polysome bound IL-7 mRNA in cumulus-surrounded oocytes in the presence of AREG, while total mRNA remained the same. As such, the changes in measured polysome-associated transcript levels were due to cumulus cell regulated transfer of mRNA to the polysome for translation and not due to increased transcription.

At least in the mouse, oocyte maturation initiates a transition from mRNA stability to instability, largely attributable to phosphorylation of the oocyte specific protein MSY2 (Medvedev et al., 2008). This subsequently triggers degradation of maternal mRNA both by 3' end deadenylation and 5' end decapping. It has been proposed that these processes occur in a regulated manner to decrease total mRNA load and time the oocyte to zygote transition (Ma et al., 2015, 2013). The rate at which mRNA degradation occurs at the 5' end may be regulated by the maternally imprinted genes *DCP1A* and *DCP2* (Ma et al., 2013), while 3' deadenylation may be regulated by *CNOT7* (Ma et al., 2015). These authors showed that by inhibiting accumulation of DCP1/2 proteins, there was less degradation of mRNA and the progression to EGA was subsequently slowed while inhibition of CNOT7 protein resulted in a 70% decrease in transcription at the 2-cell embryo stage.

Given the above, detected oocyte mRNA at any given time point potentially represents either those transcripts remaining after translation and degradation or those being stored for use during subsequent developmental stages such as fertilization and transition through EGA. However, several authors have shown that there are differentially expressed transcripts between *IVM* and *in vivo* matured oocytes (Katz-jaffe et al., 2009), GV and MII oocytes (Mamo et al., 2011) and oocytes matured *in vitro* in different conditions (Kumar et al., 2013; Ma et al., 2012; Van Hoeck et al., 2013; Watson et al., 2000). While some report this differential expression to be due to differences in cytoplasmic polyadenylation (CP) (Reyes et al., 2015) and not transcription, others disagree. Mamo et al. (2011) found 25% of 9500 bovine transcripts to be differentially expressed between GV and MII *IVM* oocytes and that 25% of these (approx. 600) were upregulated in MII oocytes. They discussed that this 25% upregulation defies the commonly held understanding that maturing oocytes are transcriptionally silent. They went on to show that these were in fact newly transcribed by culturing in the presence of α -amanitin (a transcription inhibitor) and finding that the transcripts were no longer upregulated. There are clearly divergent views on the regulation of the oocyte transcriptome.

Effects of glucose concentration on the oocyte and cumulus cell transcriptome

Hyperglycaemia, secondary to either Type I or II diabetes is a major clinical concern in women due to the increased prevalence of pregnancy complications, birth defects and ill health in the offspring. Many mechanisms have been put forward but the precise physiology mediating the downstream pregnancy-related effects of diabetes remains elusive. In attempts to uncover these mechanisms, researchers have investigated the oocyte and cumulus cell transcriptome in streptozotocin (STZ)-injected mice (in which pancreatic β cells have been destroyed) or the Akita mouse (which have genetic mutation in *Ins2* gene) both of which result in insulin-dependent diabetes. Several changes due to hyperglycaemia have been reported in cumulus cells from diabetic mice such as upregulation of apoptotic genes and downregulation of connexin expression and glucose transport (Chang et al., 2005; Colton et al., 2003; Ratchford et al., 2008; Wang et al., 2010). In 2012, Ma et al. carried out whole transcriptome analysis on oocytes from normal, STZ injected and

non-obese diabetic (NOD) mice. They reported that 535 genes were upregulated and 83 genes downregulated in both the STZ and NOD mice compared to the normal mice. During IVM, genes related to the hexosamine biosynthesis pathway (HBP) and endoplasmic reticulum stress are also upregulated in murine COCs in the face of hyperglycaemia (30 mM glucose), some after only 8 hours in culture (Wong et al., 2015). A single study in the buffalo reported differential expression of carbohydrate metabolism genes in oocytes after IVM attributable to medium glucose concentrations (0, 1.5, 5.6 and 10 mM glucose; Kumar et al., 2013).

The equine oocyte and cumulus cell transcriptome- what is known?

There have been several gene expression studies in equine oocytes and cumulus cells. Thus far, they have been limited to comparing *in vivo* maturation and IVM, the effect of mare age and the influence of maturation status and cumulus morphology (Summarised in Table 6.1). Of interest to this study, *TFAM* has been shown to be differentially expressed in oocytes, and *GPX1* and *SLC2A1* differentially expressed in cumulus cells in COCs from different sources or treatments.

There are no studies on the effect of culture conditions on the equine COC transcriptome. In previous chapters the independent effect of glucose (5.6 mM and 17 mM) and pyruvate (0 mM, 0.15 mM and 0.5 mM) on COC meiotic and developmental competence, glucose metabolism and mitochondrial function was evaluated; while there was an effect of glucose concentration on mitochondrial function, this was not associated with a significant effect on measured metabolic parameters or on meiotic or developmental competence. The mechanism by which the equine COC can tolerate and thrive in high glucose media remains unclear. DMEM/F12, one of the media used clinically for IVM in the horse, combines high glucose (17 mM glucose) and pyruvate (0.5 mM). It is possible that the supply of pyruvate in DMEM/F12 is protective or ameliorates the effect of the high glucose. In this regard, two further media are introduced to the experimental design in the current study; DMEM/F12 itself and M199 medium with added glucose (17 mM) and pyruvate (0.5 mM) to account for this difference in composition.

Genes for key factors in metabolic pathways, and enzymes of carbohydrate metabolism, known to be affected by hyperglycaemia (measured in blastocysts in Chapter 5) were measured in oocytes/cumulus cells in the present chapter. These are glycolysis (phosphofructokinase [PFK] and lactate dehydrogenase [LDHA]), pentose phosphate pathway (Glucose-6-phosphate dehydrogenase [G6PD]), glucose transporter solute carrier family 2 member 1 (SLC2A1; formerly GLUT 1) and fatty acid transporter carnitine palmitoyltransferase 1A (CPT1A). Finally, markers of oxidative stress and mitochondrial replication; glutathione peroxidase 1 (GPX1) and mitochondrial transcription factor A (TFAM) were also evaluated.

Area of Study	Oocyte	Cumulus	Type of pathway	Author
Pre and post IVM	<p>↓ <i>IL1β</i> MII</p> <p>↔ <i>ILIR2</i></p> <p>↓ <i>TFAM</i>, <i>mtPLOB</i>, <i>ATP-synthF6</i> MII</p> <p>↔ <i>SSB</i>, <i>GPX3</i></p> <p>↔ <i>KAT8</i>, <i>HAT</i>, <i>SIRT</i>, <i>HDAC1</i></p>	<p>↓ <i>GPX</i> MII</p> <p>↔ <i>Connexin 43</i>, <i>COX2</i>, <i>FSHr</i></p>	<p>Oxidative stress</p> <p>Cumulus expansion</p> <p>Inflammation</p> <p>Mitochondrial replication, oxidative stress and energy production</p> <p>Histone deacetylation</p>	<p>Luciano et al., 2006</p> <p>Dell'Aquila et al., 2004</p> <p>Martoriati et al., 2002</p> <p>Hendriks et al., 2015</p> <p>Franciosi et al., 2015</p>
In vivo vs. IVM	<p>↓ <i>IL1β</i> IVM</p> <p>↓ <i>HK16</i> acetylation IVM</p> <p>↔ <i>KAT8</i>, <i>HAT</i>, <i>SIRT</i>, <i>HDAC1</i></p>		<p>Inflammation</p> <p>Histone deacetylation</p>	<p>Martoriati et al., 2002</p> <p>Franciosi et al., 2015</p>
Mare age	<p>↓ <i>GDF9</i>, <i>BMP15</i> in old mares</p> <p>↔ <i>LHR</i>, <i>AREG</i>, <i>EREG</i>, <i>PDE</i>, <i>GPR3</i></p> <p>↔ <i>TFAM</i>, <i>GPX3</i>, <i>mtPLOB</i>, <i>ATP-synthF6</i>, <i>SSB</i></p> <p>↓ <i>IF3</i>, <i>HSF5</i>, <i>YBX2</i> in old mares</p>	<p>↔ <i>LHR</i>, <i>AREG</i>, <i>EREG</i>, <i>PDE</i>, <i>GPR3</i>, <i>GDF9</i>, <i>BMP15</i></p> <p>↑ <i>ARL6IP6</i>, <i>BAX</i>, <i>HYOU1</i> in old mares</p> <p>↑ <i>INSR</i>, <i>ADIPOR2</i>, <i>PPARG</i>, <i>IL6</i>, <i>IL6S</i>, <i>STAR</i>, <i>TXN2</i> in old mares</p> <p>↓ <i>TNF</i> old mares</p>	<p>Oocyte- cumulus communication</p> <p>Mitochondrial replication, oxidative stress and energy production</p> <p>Apoptosis, oxidative stress</p> <p>Metabolism, inflammation (*Mural granulosa only)</p>	<p>Campos-chillon et al., 2015</p> <p>Hendriks et al., 2015</p> <p>Cox et al., 2015</p> <p>Sessions-Bresnahan and Carnevale, 2015</p>
Obesity		↓ <i>PLIN2</i> , <i>EIF2AK3</i> , <i>IGFR1/2</i> in obese mares	Lipid homeostatis, oxidative and mitochondrial stress	Sessions-Bresnahan et al., 2016
Metabolic Syndrome		↓ <i>EREG</i> in EMS mares	Oocyte maturation (*Mural granulosa only)	Sessions-Bresnahan et al., 2014
Cumulus morphology	<p>↑ <i>TNFAIP6</i> in intact EX COC</p> <p>↔ <i>SLC2A1</i>, <i>PFKF</i>, <i>LDHA</i>, <i>GDF9</i>, <i>BMP15</i>, <i>FAS</i>, <i>BAX</i>, <i>BCL2L1</i>, <i>SLC2A3</i>, <i>PDK3</i></p> <p>↑ <i>TFAM</i>, <i>STAT3</i>, <i>CKS2</i> in EX</p> <p>↓ <i>COX1</i>, <i>ATPV6E</i>, <i>DNMT1</i> in EX</p> <p>↔ <i>BAX</i>, <i>p53</i>, <i>SURVIVIN</i></p>	<p>↑ <i>SLC2A1</i>, <i>TNFAIP6</i> in EX</p> <p>↓ <i>FAS</i>, <i>BAX</i> in EX</p> <p>↔ <i>PFKF</i>, <i>LDHA</i>, <i>FASLG</i>, <i>BCL2L1</i>, <i>SLC2A3</i>, <i>PDK3</i></p> <p>↓ <i>BAX</i> in EX</p> <p>↔ <i>p53</i>, <i>SURVIVIN</i></p>	<p>Cumulus expansion, glucose transport and metabolism (*intact COC vs. mural granulosa only)</p> <p>Oxidative stress, mitochondria, cell cycle</p>	<p>Gonzalez-fernandez et al., 2018</p> <p>Mohammadi-Sangcheshmeh et al., 2014</p> <p>Leon et al., 2013</p>

Table 6.1: Summary of the literature on equine oocyte and cumulus gene expression. Genes relative to this chapter are highlighted in yellow

Summary

By evaluating relative gene expression in oocytes and cumulus cells in response to hyperglycaemia during IVM, the aim was to shed light on the mechanism by which mitochondrial efficiency is altered but developmental potential and glucose metabolism are not. Uniquely, gene expression analysis allows associations between oocyte/cumulus cell relative gene expression and COC glucose, lactate and pyruvate metabolism to be explored in detail.

6.2 Objectives

1. Evaluate the impact of increased glucose (17 mM vs. 5.6 mM), increased pyruvate concentration (0.5 vs. 0 mM), and an established medium containing 17 mM glucose and 0.5 mM pyruvate, during IVM on:
 - a. Relative expression of genes associated with glucose metabolism (*G6PD*, *PFKM* and *LDHA*), oxidative stress (*GPX1* and *TFAM*), glucose transport (*SLC2A1*) and fatty acid transport (*CPT1A*) in single oocytes and corresponding cumulus cells (Experiment 6.1, Study 1).
 - g. Glucose consumption and lactate and pyruvate production rates of COCs during IVM (Experiment 6.1, Study 2).
2. Explore associations between the relative expression of the above genes in oocytes and their corresponding cumulus cells (Experiment 6.1, Study 3A).
3. Explore associations between the relative expression of individual genes in the same oocyte and cumulus cell sample (Experiment 6.1, Study 3B).
4. Explore the association between the relative expression of the above metabolic genes in the oocyte and cumulus cells and the corresponding COC metabolism during IVM (Experiment 6.1, Study 3C).

6.3 Materials and Methods

COCs were recovered from abattoir-derived ovaries, classified and held overnight as previously described in Sections 2.1.1 and 2.1.2. Compact (Cp) and expanded (Ex) COCs were divided equally and randomly between conditions, so that for each collection day, each maturation condition contained equal numbers of each cumulus type.

In vitro maturation

Maturation was carried out in individual 10- μ l droplets at 5% CO₂ in air. There were 12 droplets in each dish; COCs were placed in nine of them while three remained empty and were used as reference droplets. Dishes were continuously incubated for the entire 30-hour IVM period in one of four IVM conditions as follows: 1) Control Maturation Medium (5.6 mM glucose; M199 with Earle's salts, 10 % FBS, 25 μ g/ml gentamicin with 5 mU/ml FSH; 0 mM pyruvate) [**Group C**]; High glucose Maturation Medium (17 mM glucose; As above with addition of glucose) [**Group HG**]; DMEM/F12 Maturation medium (17 mM glucose and 0.5mM Pyruvate; DMEM/F12 [Sigma], 10 % FBS, 25 μ g/ml gentamicin with 5 mU/ml) [**Group DMEM**]; High glucose, high pyruvate Maturation Medium (high glucose [17mM] maturation medium as above with the addition of 0.5 mM pyruvate) [**Group HG-HP**]. This medium was chosen due to the many differences in composition between the DMEM/F12 and M199 media other than the glucose/pyruvate concentrations.

After the maturation period was complete, COCs were denuded and classified as Metaphase II, intact or degenerated as previously described in Section 2.1.4. Denuded cumulus cells from each MII COC were placed in a labelled 96-well plate and kept at -20 °C until later DNA quantification analysis. Some denuded cells were saved for extraction of mRNA. Spent media were frozen at -80° C and analyses of thawed samples carried out as previously described in Section 2.2.

Gene expression analysis

In each of the three replicates, cDNA was prepared from mRNA extracted from two immature oocytes with compact cumulus (Cp) and two immature oocytes with expanded cumulus (Ex). After maturation, cDNA was prepared from all MII oocytes and from a sample of each oocyte's corresponding cumulus cells (those associated with the oocyte during IVM and removed by denuding). Lysis, cDNA amplification and picogreen quantification were carried out as detailed in Section 2.5.1.

Seven genes of interest were investigated and Taqman® probe based primers designed within 300 bp of the polyA tail at the 3' UTR (Primerdesign LTD; Table 6.2). All primers were the same as those used for the blastocysts in Chapter 5 and optimised and tested using amplified cDNA of each sample type by Primerdesign LTD.

Name	Accession number	Sense primer	Antisense primer
G6PD	XM_001492232	CTTGCCACTAGGAAAGTA GAAGC	AGGCAGGTCTGTTTGTGGA TT
PFKM	NM_001081922	TGGAAGCTCCTTTTAGGT AGAATTAT	AATCTCGCAGTGGCACTAG AGG
Cpt1A	NM_001081808	TTCCTCCATCCAGGGTTG G	GACCTGCCCAGAAGTAGTA GG
GPX1	NM_001166479	GAACGCCTGATGTTAAGG AGAAT	TTATTAGTGAGAAGCCGTG GTCT
TFAM	XM_001503382.3	ATCTTGGAAGAACAAT GATTGAAG	TGTCTATCTACTGTGAACAT AACTCAA
LDHA	NM_001144880	GATGCGTGTTTACTCTGT GTGAT	TCTCAAGATGTGACTGACT GAAGA
SLC2A1	NM_001163971	GAGGTCAGGCTCCATTAG GATT	CAACTGGTCTCAGGCAAGG A

Table 6.2: Genes and primer sequences for genes of interest used in Study 1.

qPCR reactions were made up as follows and plated in triplicate; 5 µl cDNA (5ng), 1 µl primer, 10 µl PrecisionPlus mastermix (Primerdesign LTD) and 4 µl PCR water. Amplification was performed on a Biorad CFX using the following protocol; initial enzyme denaturation of 2 minutes at 95 °C then 50 cycles of 15 seconds at 95 °C

and 60 seconds at 60 °C. Data was collected through the FAM channel at the final stage. Reference genes RPL32 and UBC were also run for each sample on each plate for further stability testing (See Section 2.5.2).

Statistical analysis

Study 1

The rate of maturation to MII was compared among treatments using the Chi-square test. The mean Ct value from each technical triplicate was calculated for each sample and retained if variation was < 1 Ct. Ct values over 36 with high technical variation were assumed to be not expressed. ΔCt was then calculated for each sample by subtracting the Ct value of the reference gene of that sample (from that plate) from the Ct value of the gene of interest. Relative expression ($2^{-\Delta Ct}$; Schmittgen and Livak, 2008) was then compared across experimental groups using either Student's t-test (immature vs. MII) or One-way ANOVA using the Bonferroni statistic.

Study 2

The primary outcome variables were glucose consumption, lactate and pyruvate production rates (expressed as pmol/ngDNA/hour). One-way ANOVA using the Bonferroni statistic was used to explore differences related to IVM condition.

Study 3

Relative gene expression between oocytes and corresponding cumulus cells (Study 3A) and between individual genes (Study 3B) were analysed by linear regression. Relative gene expression for both oocytes and cumulus cells was analysed by linear regression against the corresponding COC glucose consumption, lactate, and pyruvate production rates (all expressed as pmol/ngDNA/hour) and the ratio of lactate production to glucose consumption during IVM (Study 3C).

6.4 Results

Twelve immature oocytes and 61 IVM oocytes were evaluated over three replicates. Of the IVM oocytes, 26 (42.6%) reached Metaphase II. There was no significant difference in maturation rate among IVM treatments ($P > 0.1$). For 5/26 MII COCs there were insufficient numbers of cumulus cells for both DNA quantification and mRNA extraction, in these cases (where there were enough cells) mRNA extraction was prioritised and spent media analyses were not performed for those COCs. Where there were insufficient cells for either evaluation, only the MII oocyte was used for mRNA extraction (see Table 6.3).

	Immature	Control (5.6 mM G/ 0 mM P)	High Glucose (17 mM G/ 0 mM P)	High Glucose High Pyruvate (17mM G/ 0.5mM P)	DMEM (17 mM G/ 0.5 mM P)	Total
n	12	14	14	15	18	73
MI I %	N/A	43% (6/14)	50% (7/14)	27% (4/15)	50% (9/18)	26
GPL	N/A	4	7	3	7	21
cDNA oocytes	11	6	7	4	9	37
cDNA cumulus	10	5	7	4	7	33

Table 6.3: Number of COCs assigned to each experimental group (n). Maturation rate for each experimental condition and number of samples subsequently available for each study is also shown (GPL; glucose pyruvate lactate metabolic measurements).

6.4.1 Experiment 6.1, Study 1: Effect of increased glucose and pyruvate concentration during IVM on gene expression

Cumulus cells

Thirty-three cumulus cell samples were analysed in total across the experimental groups and all data were included in the analysis (Table 6.3). As each housekeeping gene was plated for each sample on each plate, the inter-plate variation could be calculated. This was $\leq 1\%$. Reference genes *UBC* and *RPL32* were further examined for suitability for normalisation, and as *RPL32* showed higher expression in all samples (mean Ct 19.5 vs. 23.4 for *UBC*) and also less variation (SD 2.9 and CV 15% vs. 4.8 and 20% for *UBC*), *RPL32* was used to calculate ΔCt values.

Across all groups, *GPX1*, *LDHA* and *G6PD* showed the highest relative expression and were detected in all samples (Figure 6.1). One gene, *PFKM*, was differentially expressed, being significantly higher in cumulus from MII oocytes than in cumulus from immature oocytes ($p < 0.05$).

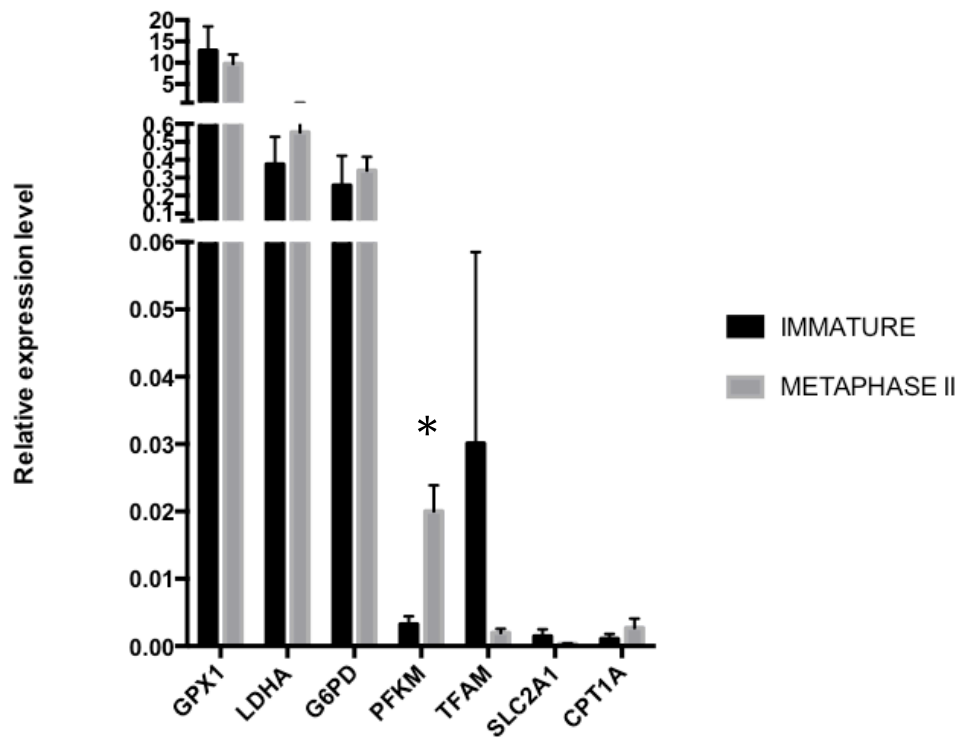


Figure 6.1: Summary of relative expression (normalised against RPL32) of each gene in cumulus cells across all experimental groups. $N = 33$. Mean \pm SEM are presented. Asterisk represents significant difference ($p < 0.05$).

As each group contained a similar number of oocytes with Ex and Cp cumulus cells, relative gene expression data for each gene of interest were first compared between Ex and Cp within each condition (Immature [IMM], control maturation media [C], high glucose maturation media [HG], high glucose high pyruvate maturation media [HG-HP] and DMEM media). In Group DMEM, Ex showed higher *G6PD* expression than did Cp ($p < 0.01$). In group HG-HP, *LDHA* showed higher expression in Ex compared to Cp ($p < 0.01$). Of the thirty comparisons, there were no other differences attributable to Ex/Cp cumulus classification and, notably, none existed within the immature (pre-maturation) samples or when conditions were merged and so for further analysis Ex and Cp were treated as one group.

When differences among IVM treatments were evaluated, expression of *G6PD* was found to be significantly higher in Group HG-HP compared to HG ($p < 0.05$; Figure 6.2). Biological variation was large within experimental groups and no other significant differences were detected in relative gene expression (Figure 6.2, 6.3, 6.4). Two of the four COCs in the control group depleted all available glucose during IVM and are highlighted separately.

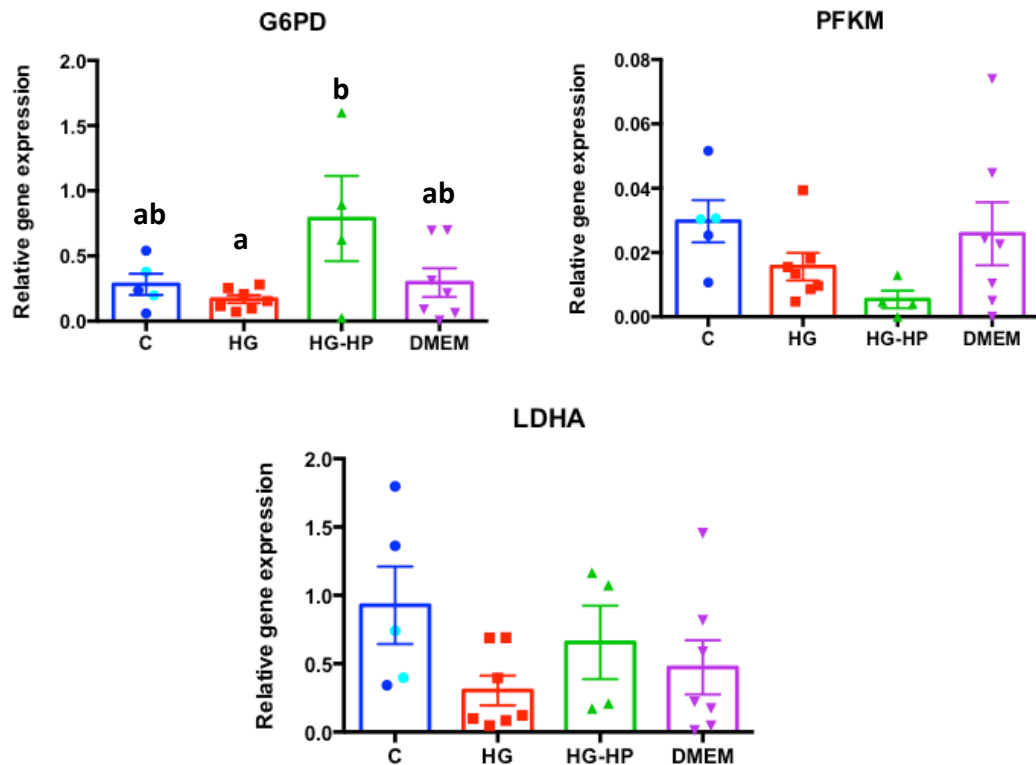


Figure 6.2: Relative gene expression (normalised against *RPL32*) of genes related to glucose metabolism in the cumulus cell samples. Experimental groups abbreviated as follows; control maturation media [C; BLUE, COCs that depleted all available glucose during IVM are highlighted in light blue], high glucose maturation media [HG; RED], high glucose high pyruvate maturation media [HG-HP; GREEN] and DMEM media; PINK. Mean \pm SEM are presented with significance differences between groups indicated by different superscripts for *G6PD* ($p < 0.05$).

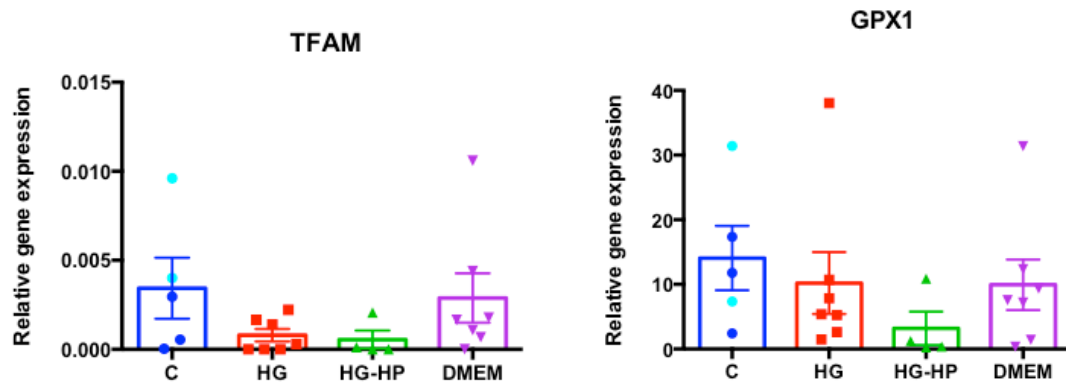


Figure 6.3: Relative gene expression (normalised against RPL32) of genes related to oxidative stress in the cumulus cell samples. Experimental groups abbreviated as follows; control maturation media [C; BLUE, COCs that depleted all available glucose during IVM are highlighted in light blue], high glucose maturation media [HG; RED], high glucose high pyruvate maturation media [HG-HP; GREEN] and DMEM media; PINK. Mean \pm SEM are presented.

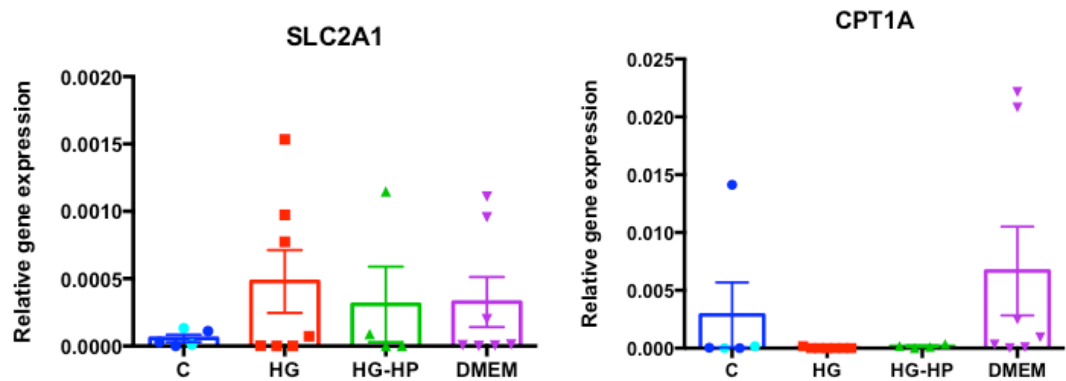


Figure 6.4: Relative gene expression (normalised against RPL32) of genes related to substrate transport in the cumulus cell samples. Experimental groups abbreviated as follows; control maturation media [C; BLUE, COCs that depleted all available glucose during IVM are highlighted in light blue], high glucose maturation media [HG; RED], high glucose high pyruvate maturation media [HG-HP; GREEN] and DMEM media; PINK. Mean \pm SEM are presented.

Oocytes

Thirty-seven individual oocytes (11 immature and 26 MII) were analysed in total across the experimental groups. One oocyte (Group HG-HP) was excluded due to lack of expression of all target genes and reference genes. The inter-plate variation was $\leq 1\%$. Reference genes *UBC* and *RPL32* were further examined for suitability for normalisation, and as *UBC* showed higher expression in all samples (mean Ct 20.8 vs. 23.9 for *RPL32*) and also less variation (SD 3.6 and CV 17% vs. 45.3 and 22% for *RPL32*), *UBC* was used to calculate ΔCt values. Across all groups, as for cumulus cells, *GPX1*, *LDHA* and *G6PD* showed the highest relative expression (Figure 6.5). Only *GPX1* was universally expressed; 92%, 94%, 25%, 11% and 28% of samples showed expression of *LDHA*, *G6PD*, *PFKM*, *TFAM* and *SLC2A1* respectively. *CPT1A* was not detected in any sample. *TFAM* had higher relative expression in immature oocytes than in MII oocytes ($p < 0.05$) and *LDHA* tended to have higher relative expression in immature oocytes than in MII oocytes ($p = 0.06$).

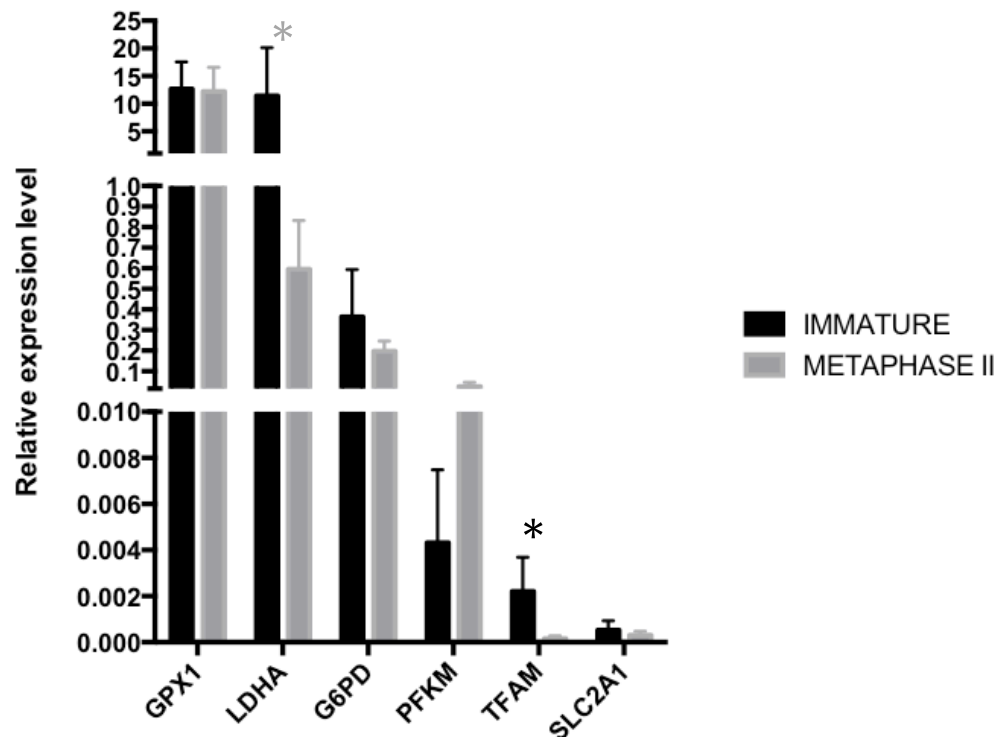


Figure 6.5: Summary of relative expression (normalised against *UBC*) of each gene in oocytes across all experimental groups. $N = 37$. Mean \pm SEM are presented. Black asterisk represents significant difference ($p < 0.05$) and grey asterisk $p=0.06$.

As each group contained a similar number of Ex and Cp oocytes, relative gene expression data for each gene of interest were first compared between Ex and Cp within each condition. *GPX1* showed higher relative expression in Cp compared to Ex in Group HG ($p < 0.05$). There were no other differences attributable to cumulus classification so for further analysis they were treated as one group.

Biological variation was large within experimental groups and no significant differences were detected in relative gene expression attributable to experimental condition ($p > 0.05$; Figure 6.6). Two of the four COCs in the control group depleted all available glucose during IVM and are highlighted separately.

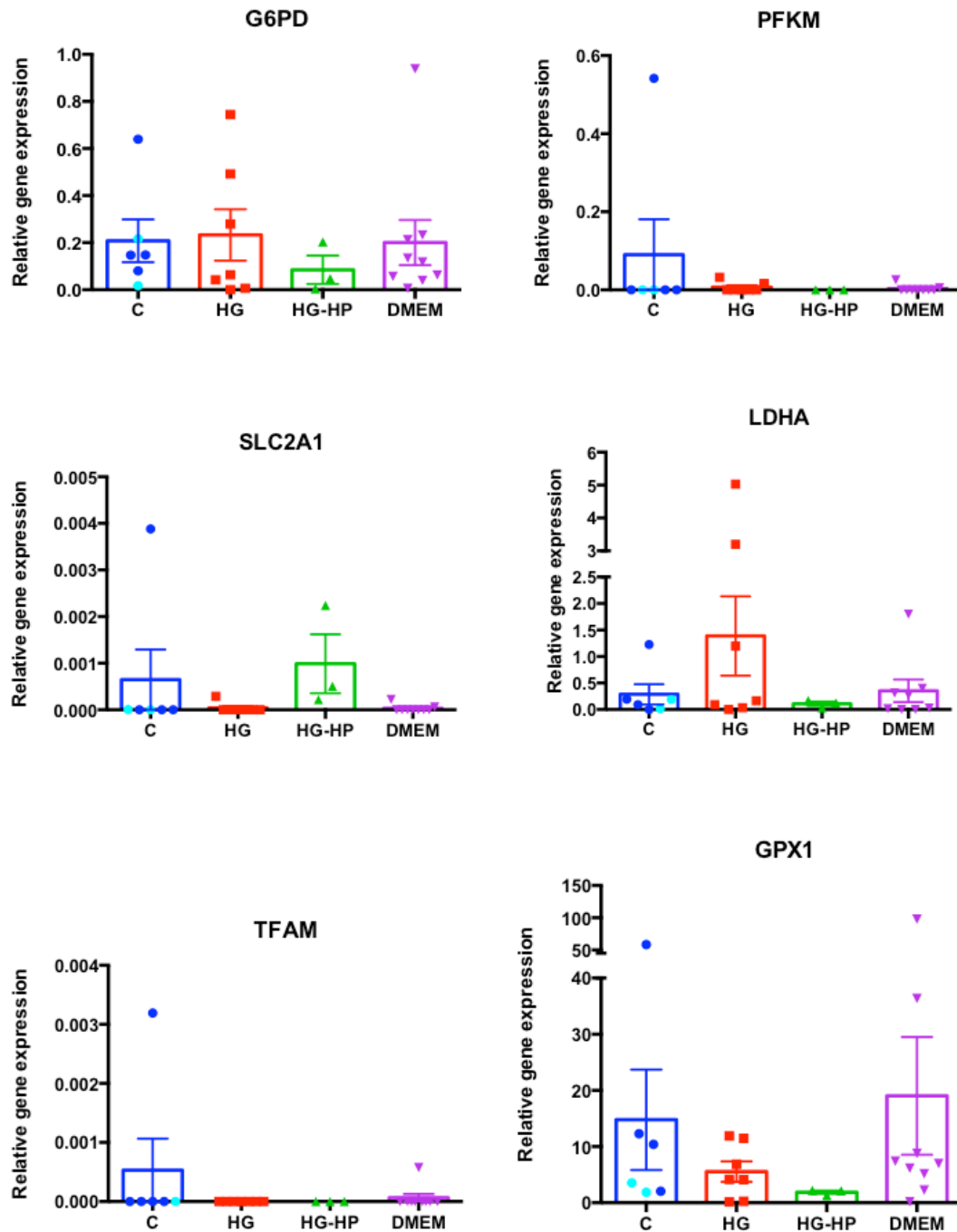


Figure 6.6: Relative gene expression (normalised against UBC) of genes investigated in the oocyte samples. Experimental groups abbreviated as follows; control maturation media [C; BLUE, COCs that depleted all available glucose during IVM are highlighted in light blue], high glucose maturation media [HG; RED], high glucose high pyruvate maturation media [HG-HP; GREEN] and DMEM media; PINK. Mean \pm SEM are presented where appropriate.

6.4.2 Experiment 6.1, Study 2: Effect of increased glucose and pyruvate concentration during IVM on COC metabolism.

Numbers available for metabolic analysis were low. For 5/26 COCs there were insufficient numbers of cumulus cells for both DNA quantification and mRNA extraction; in these cases (where there were enough cells) mRNA extraction was prioritised and spent media analyses were not performed (Table 6.5). Two of the four COCs in Group C depleted all available glucose and were not included in GPL analysis.

	Control (C) (5.6 mM G/ 0 mM P)	High Glucose (HG) (17 mM G/ 0 mM P)	High Glucose High Pyruvate (HG-HP) (17mM G/ 0.5mM P)	DMEM (17 mM G/ 0.5 mM P)	Total
MII oocytes	6	7	4	9	26
GPL	4	7	3	7	21

Table 6.4: Number of oocytes that reached MII in each condition and number of COCs for which glucose, pyruvate, lactate (GPL) analysis of spent media was performed.

There were no significant differences in glucose consumption or lactate production rates between groups. Pyruvate was consumed in both HG-HP media and DMEM media (both containing 0.5 mM pyruvate) whereas it was produced in the other two conditions in which pyruvate was not present ($p < 0.05$; Figure 6.7).

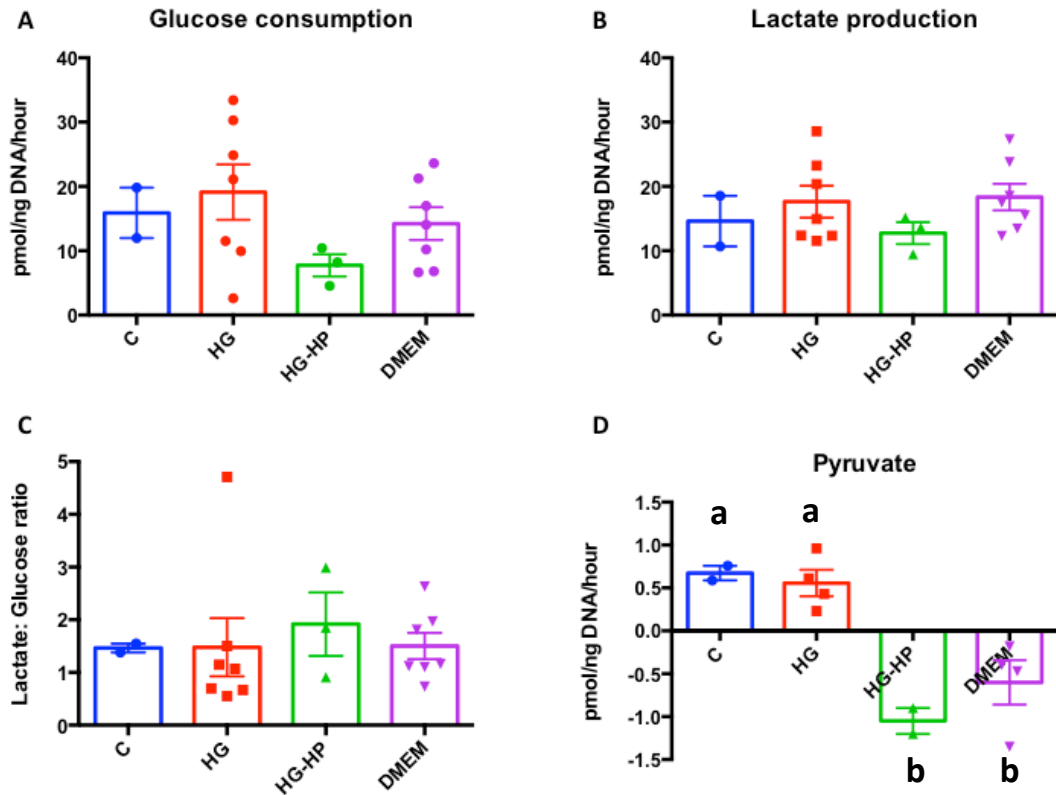


Figure 6.7: Effect of media on glucose consumption (A), lactate production (B), lactate: glucose ratio (C) and Pyruvate production/consumption (D). Experimental groups abbreviated as follows; control maturation media [C; BLUE], high glucose maturation media [HG; RED], high glucose high pyruvate maturation media [HG-HP; GREEN] and DMEM media; PINK. Mean \pm SEM are presented and different superscripts represent significance difference between groups.

6.4.3 Experiment 6.1, Study 3A: Association between gene expression in oocytes and corresponding cumulus cells

Relative gene expression of all six genes was compared between the oocyte and corresponding cumulus cells for the 32 COCs for which both expression data existed (data not shown). Only *GPX1* expression showed a weak association ($p = 0.16$; Figure 6.8). There were no further associations between the relative expression of any gene in oocytes and their corresponding cumulus cells ($p > 0.05$)

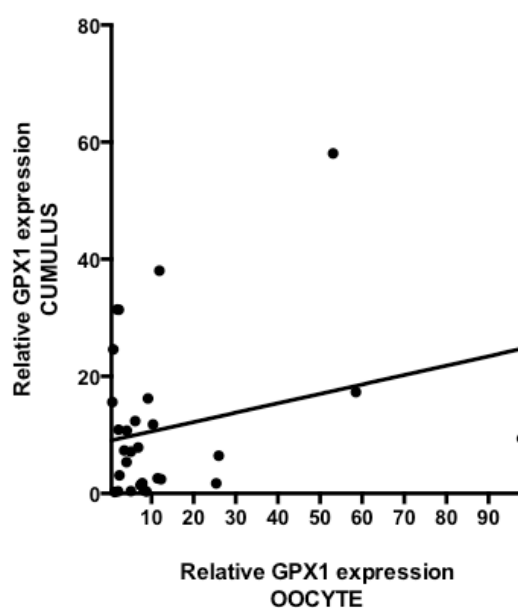


Figure 6.8: Correlation between relative *GPX1* expression in oocytes and their corresponding cumulus cells ($p = 0.16$; $r^2 = 0.06$).

6.4.4 Experiment 6.1, Study 3B: Association between relative expression of individual genes

Cumulus cells

Forty-two pairwise linear regressions were performed to explore associations between relative expression of individual genes (data not shown). *G6PD* expression was positively correlated to *GPX1* expression ($p < 0.001$; $r^2 = 0.3$; Figure 6.9A) and both *G6PD* and *GPX1* were positively correlated to *TFAM* ($p < 0.001$; $r^2 = 0.4$; Figure 6.9B and 6.9C). *LDHA* was also positively correlated to *PFKM* expression ($p = 0.03$; $r^2 = 0.15$; Figure 6.9D).

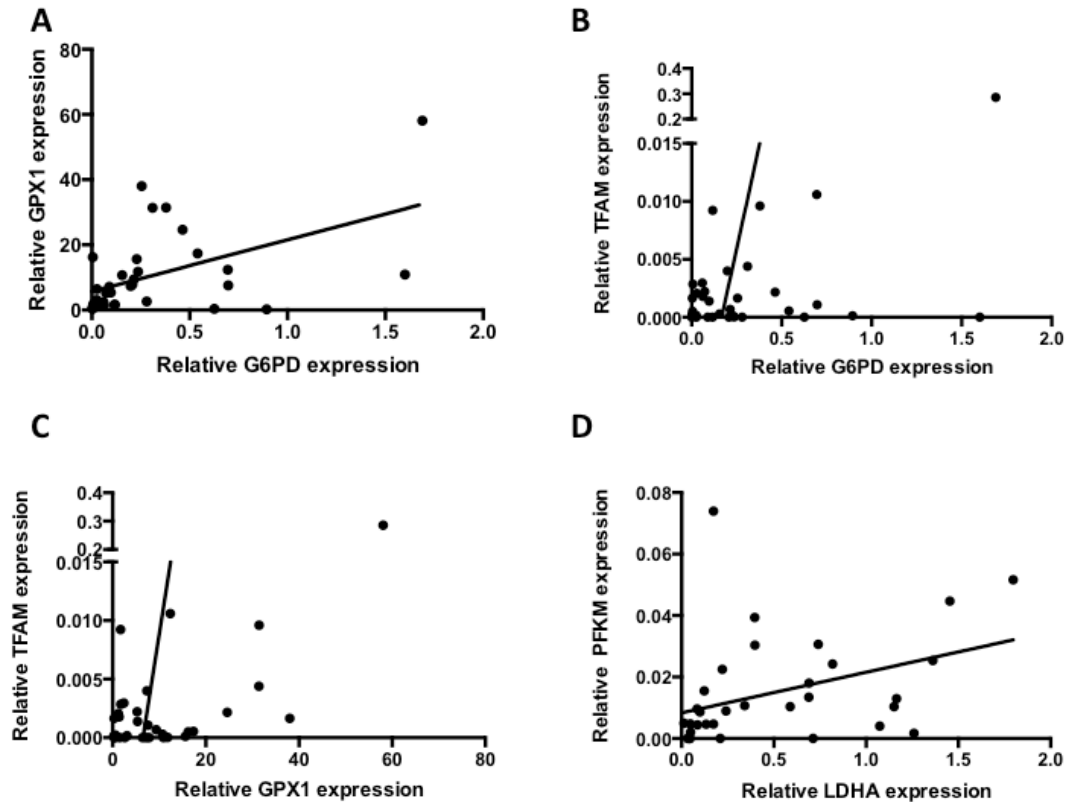


Figure 6.9: Correlation between *G6PD* expression and *GPX1* (A; $p < 0.001$; $r^2 = 0.3$), *G6PD* and *TFAM* (B; $p < 0.001$; $r^2 = 0.4$), *GPX1* and *TFAM* (C; $p < 0.001$; $r^2 = 0.4$), and between *LDHA* and *PFKM* ($p = 0.03$; $r^2 = 0.15$) expression in cumulus cells.

Oocytes

Thirty-six pairwise linear regressions were performed to explore associations between relative expression of individual genes (data not shown). *PFKM* expression was positively correlated to both *GPX1* expression ($p = 0.01$; $r^2 = 0.15$) and *SLC2A1* expression ($p < 0.001$; $r^2 = 0.4$), however these relationships were driven by a single oocyte.

6.4.5 Experiment 6.1, Study 3C: Associations between relative gene expression and COC metabolism during IVM

In cumulus cells, increased expression of *GPX1* was positively correlated with glucose consumption (Figure 6.10A; $p = 0.04$; $r^2 = 0.2$) and tended to be positively correlated to pyruvate production (Figure 6.10B; $p = 0.1$; $r^2 = 0.2$). In addition, *TFAM* expression was positively correlated to pyruvate production (Figure 6.11B; $p = 0.04$; $r^2 = 0.3$) and tended to be negatively correlated to lactate: glucose ratio (Figure 6.11A; $p = 0.09$; $r^2 = 0.13$). In oocytes, there were no associations between relative gene expression and any metabolic parameter measured ($p > 0.05$).

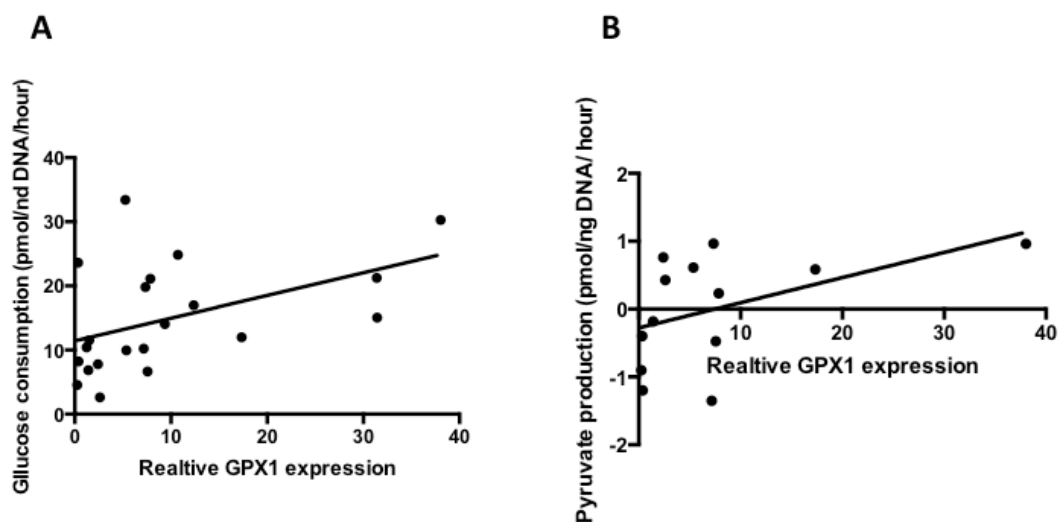


Figure 6.10: Correlation between relative *GPX1* expression in cumulus cells and glucose consumption (A; $p = 0.01$; $r^2 = 0.3$) and pyruvate production (B; $p = 0.1$; $r^2 = 0.2$).

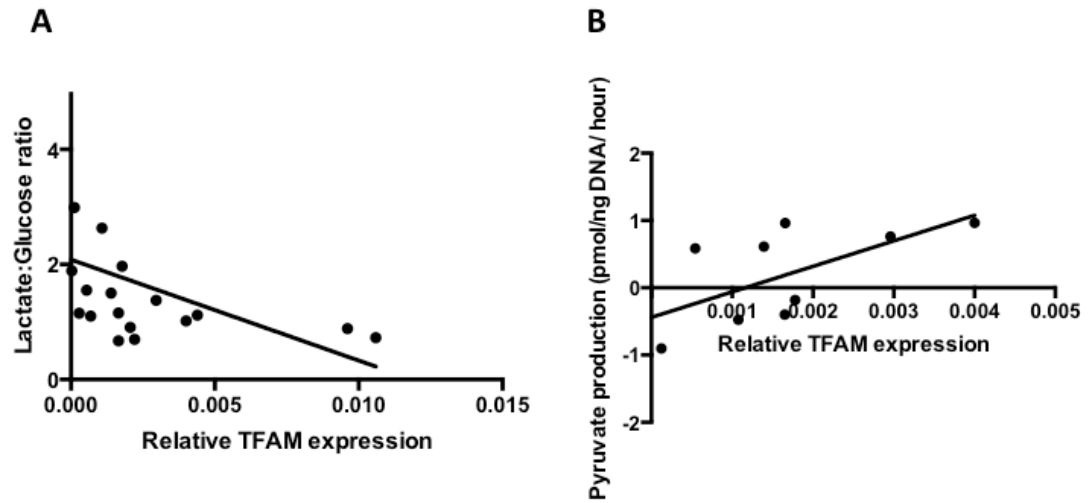


Figure 6.11: Correlation between relative TFAM expression and lactate: glucose ratio (A; $p = 0.09$; $r^2 = 0.13$) and pyruvate production (B; $p = 0.04$; $r^2 = 0.3$).

6.5 Discussion

This chapter reports the successful application of PolyA PCR to equine cumulus cells and single oocytes. The relative expression of seven genes is described. In cumulus cells, one of the seven genes, *G6PD*, was differentially expressed: this was higher in HG-HP (17 mM glucose and 0.5 mM pyruvate) than in HG (17 mM glucose and 0 mM pyruvate). Biological variation was large and as such no differences were detected in gene expression attributable to experimental condition in oocytes.

G6PD is a regulatory enzyme of the pentose phosphate pathway (PPP), important for redox balance through the production of NADPH, involved in redox status, and production of PRPP, an essential substrate for *de novo* purine synthesis. The difference observed in *G6PD* expression suggests higher flux through the PPP in group HG-HP. It is possible that the presence of pyruvate in the medium could be sparing glucose and increasing availability for the PPP. Consistent with this notion, in the presence of pyruvate, COCs shifted from pyruvate production and secretion to pyruvate consumption. As discussed in previous chapters, pyruvate secretion is often prioritised as an antioxidant defence strategy (O'Donnel Turmey et al., 1987). Interestingly, at 0.15 mM pyruvate, as used in previous experiments, pyruvate production was maintained suggesting there is a threshold between 0.15- 0.5 mM at which the shift to consumption occurs. Based on research in the preimplantation mouse embryo, which reported a requirement for an absolute medium concentration of 0.3 mM pyruvate (O'Fallon and Wright 1996), it would be interesting to test that concentration in future studies. It would also be interesting to investigate if 0.5 mM pyruvate would have a similar effect in 5.6 mM glucose medium or if the increase in PPP is only due to a synergism with high glucose.

G6PD was the third most abundant transcript in the current study, expressed in 100% of cumulus samples and 94% of oocytes. *G6PD* activity has received much attention in immature oocytes due to the use of the Brilliant cresyl blue stain (BCB). BCB is degraded by *G6PD* and as such, stained COCs become colourless when *G6PD* activity is high but stay blue when activity is low (Mohammadi-Sangcheshmeh et al.,

2011; Torner et al., 2008). The studies by Mohammadi-Sangcheshmeh et al. and Torner et al. reported that in immature equine and bovine oocytes low G6PD activity was correlated with expanded cumulus morphology (Ex) and higher meiotic and developmental competence. However, no correlation was found between G6PD activity and follicular status in another equine study (Vernunft et al., 2013). There is dynamic regulation of G6PD due to the role of the PPP in redox balance. A study in mice demonstrated that inhibiting the PPP not only resulted in a decreased blastocyst development rate but also a decrease in glutathione and NADPH content, and increased ROS (Xie et al., 2016). As described in Chapter 5, there is negative feedback on G6PD as a result of increased HBP and Poly-o-L flux in the face of hyperglycaemia. In this regard, Colton et al. (2003) reported decreased flux through the PPP in COCs from diabetic mice using radiolabelled glucose, and a study in buffalo, demonstrated a decrease in *G6PD* expression in oocytes in response to increased glucose concentration during IVM (10 mM glucose vs. 5.6 mM glucose; Kumar et al., 2013). Interestingly, in the buffalo study, low glucose concentration (0 and 1.5 mM) also resulted in decreased *G6PD* expression. There were no differences in transcript abundance in the current study in either oocytes or cumulus cells attributable to glucose concentration alone. This could be due to lack of correlation between transcript levels and enzyme activity, medium composition or high biological variation.

GPX1 was the only gene to be universally expressed in both sample types and also showed the highest relative expression in all samples. In cumulus cells *GPX1* expression was positively associated with *G6PD* and *TFAM* expression. Glutathione peroxidases are potent antioxidant enzymes, *GPX1* being the most abundant cytosolic isoform. The enzymes break down H_2O_2 to oxygen and water and thus are central in antioxidant defence and abundant in oocytes and embryos (Agarwal et al., 2012). Increased *GPX1* expression in oocytes has been correlated with decreased DNA fragmentation in pigs and large healthy follicles in cattle (Ceko et al., 2015; Whitaker and Knight, 2008). In human cumulus cells, increased *GPX1* expression was associated with positive pregnancy outcome after ICSI (Ceko et al., 2015). In the horse, *GPX1* expression has been measured in oocytes and cumulus by

Luciano et al., (2006) who found that while expression increased over the course of IVM in cumulus cells, *GPX1* mRNA was not detected in either GV or MII oocytes. Similarly, Hendriks et al., (2015) could not detect *GPX3* mRNA in 30/48 pooled equine oocyte samples. Interestingly, in a human study by El Mouatassim et al. (1999), *GPX1* mRNA was detected only in MII oocytes, however when a 3' directed primer was used instead of an Oligo based primer during reverse transcription (which recognizes the polyA tail of mRNA), *GPX1* mRNA was also detected at the GV stage. While this suggests *GPX1* mRNA is polyadenylated during maturation, in the present study, an Oligo based primer was used and as such confirms polyadenylated mRNA (of at least 24 bp) in both GV and MII equine oocytes. This could be a species difference but it is also possible that environmental factors could be responsible for such differences. For example, hyperglycaemia and subsequent increased oxidative state in humans can influence *GPX1* expression, since decreased *GPX1* in endothelium is correlated with diabetes (Hamanishi et al., 2004).

Since individual oocytes were tracked throughout IVM and mRNA extraction, associations between relative gene expression and COC glucose, lactate and pyruvate metabolism could be explored for the first time. While the coefficients of determination (r^2) were low in many of the models, the low p values show that there is still predictive value in the variables tested and as such findings will be discussed below in context of the existing literature. In cumulus cells, the expression of *GPX1* was positively correlated with glucose consumption and pyruvate production. It was shown in Chapter 3 that increased glucose consumption in COCs was associated with increased oxidative phosphorylation. Taken together these findings suggest that COCs that are more metabolically active also have a higher anti-oxidant capacity. Which of these is the main determinant cannot be ascertained from the present data, though it is interesting to speculate that the increased metabolic activity resulted in increased *GPX* transcription to protect from the inevitable increase in ROS. This is supported by the correlation with pyruvate production, also potentially as a protective mechanism against ROS. Conversely, COCs with increased antioxidant capacity could be those in which a high level of glucose and oxidative metabolism can be sustained.

TFAM was the only other gene in which the relative expression in cumulus cells was associated with COC metabolism. *TFAM* is involved in mitochondrial biogenesis and mtDNA transcription and translation (Itami et al., 2015) and expression of the gene is increased when mitochondrial regeneration or synthesis is occurring. In the present study, *TFAM* was expressed in 75% of cumulus cell samples but only 11% of oocytes. *TFAM* expression has previously been described in immature equine oocytes by Mohammadi-Sangcheshmeh et al., (2014, 2011) who reported higher *TFAM* expression in Ex oocytes (compared to Cp) and in those with low G6PD activity (BCB+) and higher developmental competence. *TFAM* expression can also be regulated by environmental glucose concentration. Specifically, in humans, *TFAM* expression is decreased in both hepatocytes and cardiomyocytes in hyperglycaemic conditions such as diabetes (Palmeira et al., 2007; Suarez et al., 2008). The study by Suarez et al., (2008) also demonstrated that by overexpressing *TFAM* using plasmid transfection, mitochondrial function was restored in cardiomyocytes. In the oocyte, the mechanisms surrounding mitochondrial biogenesis and repair are poorly understood; however Itami et al., (2015) demonstrated that porcine oocytes had the capacity for mitochondrial regeneration following FCCP-induced damage, as indicated by rising *TFAM* mRNA levels after prolonged culture. This mechanism was subsequently shown to be affected by advance age in the cow: recuperation was not as pronounced in oocytes from aged animals after FCCP treatment than in those from young animals (Kansaku et al., 2017). In a murine study, an unexpected finding was that *TFAM* mRNA levels and mtDNA copy number were increased in cumulus cells from diabetic mice (Wang et al., 2010). As suggested above, the authors speculated that this was a compensatory mechanism in response to the decreased efficiency of individual mitochondria. It is interesting that both cumulus cell samples from COCs that had depleted all available glucose during IVM (2/6 depleted in Group C) expressed *TFAM* outside the mean and higher than all other samples.

TFAM expression in cumulus cells was negatively associated with lactate : glucose ratio and positively associated with pyruvate production. As the lactate : glucose ratio allows an estimate of the proportion of glucose consumed by glycolysis rather than other pathways, the current data suggest that as glycolysis proportionately decreased, *TFAM* expression increased. Again, the mechanisms involved are not clear however if more OXPHOS was occurring it could feed back to initiate mitochondrial transcription especially if OXPHOS was inefficient as suggested by Wang et al., (2010), or alternatively the presence of more mitochondria may be able to sustain a higher rate of OXPHOS. The mechanism behind the association with pyruvate production is unclear, especially as pyruvate production was linked with the absence of pyruvate in the medium.

Given the associations between *GPX1* and *TFAM* expression in cumulus cells and metabolic activity of the COC, it is notable that there were no such associations in oocytes. This supports the theory that transcript abundance is under post-transcriptional control and as such, the amount of mRNA is not closely linked with enzyme activity. The other explanation is that the oocyte itself and its metabolic enzymes contribute very little to the metabolic activity of the COC. It is likely to comprise a combination of both explanations, however as >70% samples had no expression *PFK*, *TFAM*, *SLC2A1* and none had *CPT1A*, more experiments would be required to uncover the mechanisms involved. The low expression of *PFK* and *SLC2A1* is in agreement with the current accepted understanding that the oocyte itself has little capacity to metabolise glucose; cumulus cells provide the oocyte with pyruvate through glycolysis (Cetica et al., 2002; Downs and Utecht, 1999; Figure 1.9). The finding that glycolytic enzyme genes *LDHA* and *PFK* expression were correlated in cumulus cells is therefore not surprising.

Murine studies investigating the effects of hyperglycaemia have reported a clear and consistent decrease in glucose uptake by cumulus cells concurrent with decreased expression of glucose transporters (Wang et al., 2012, 2010). In the present study, while the numbers for comparison were small, equine COCs did not appear to respond to hyperglycaemia (17 mM) by decreasing expression of *SLC2A1*.

While a decrease in transcription is only one of the mechanisms by which glucose transport can be reduced, this is in agreement with the unchanged glucose consumption reported herein and in Chapter 5. The reason for this is unclear, however perhaps examining the expression of other glucose transporters and exposing equine COCs to even higher glucose concentrations would aid in deciphering the underlying physiology. It is interesting to speculate that a lack of downregulation of glucose transport could be central to the mechanism by which equine COCs tolerate high glucose concentrations. In mice, it has been proposed by Wang and Moley, (2010) that the deficiency in cumulus cell glucose transport initiates the series of events leading to a decline in oocyte quality. As cumulus cells are central to providing pyruvate and ATP to the oocyte, decreased glucose transport results in decreased pyruvate and ATP transfer, since such oocytes from diabetic mice contain less ATP. Additionally, lack of glucose transport can trigger BAX dependent apoptotic pathways (Chi et al., 2000). It has been postulated by the same authors (Wang and Moley) that toxic factors related to apoptosis such as cytochrome C and excess calcium, could then be transferred to the oocyte and disrupt a myriad of cytoplasmic functions. None of these events would occur if glucose transport were maintained.

The lack of *CPT1A* mRNA in equine oocytes is interesting as it has been detected in bovine oocytes by Van Hoeck et al., (2013). Carnitine palmitoyl transferase 1 (CPT1) is the enzyme responsible for the transfer of fatty acids across the outer mitochondrial membrane and is the rate-limiting step of β -oxidation. CPT1 has three isoforms 1A, 1B and 1C. Both *CPT1B* and *1A* mRNA have been detected in the mouse however whole COCs were analysed rather than denuded oocytes (Dunning et al., 2014a, 2010). As β -oxidation of fatty acids likely plays a role in equine oocyte maturation, this result highlights the need for caution in interpretation, as detected transcripts levels often do not correlate well with enzyme activity. This was demonstrated in a study by Forsey et al., (2013) when investigating the role of creatine kinase (CK) in the murine preimplantation embryo. In that study, no correlation existed between CK mRNA levels and measured CK enzyme activity.

In the present study, there was higher relative abundance of *TFAM* mRNA in immature oocytes compared to MII oocytes. In agreement with this finding, Hendriks et al., (2015) found that *TFAM* expression was decreased in MII equine oocytes compared to immature oocytes. As *TFAM* expression is closely linked to mtDNA copy number, it is notable that Rambags et al., (2014) reported a decrease in equine mtDNA copy number after IVM but only in aged mares. This was associated with loss of normal mitochondrial architecture. It is accepted in other species and recently demonstrated in the horse by Hendriks et al., (2018) that mitochondrial replication ceases as MII is reached and that the resulting mitochondrial complement is relied on through early development until the blastocyst stage (Review; St. John, 2012). The decrease in *TFAM* mRNA levels after IVM is in keeping with these findings.

Conclusions

The aim of this chapter was to use transcriptomics of oocytes and cumulus cells to provide further insight into the effect of high glucose during IVM. Our main finding was that presence of 0.5 mM pyruvate in high glucose medium was associated with a switch to pyruvate consumption rather than production and with an increase in *G6PD expression*, an enzyme of the PPP pathway. Decreased *TFAM* expression was also demonstrated in MII oocytes compared to immature oocytes. In addition, the necessary methods were refined and validated by proof of principle in single oocyte/COC analysis, creating a platform for further research to be performed. Findings were consistent with independent regulatory mechanisms of the oocyte and cumulus cell transcriptome and lack of association between the oocyte transcriptome and COC metabolism, adding to current understanding of the physiology of oocyte cumulus cell interactions.

7. General discussion

Assisted reproduction technologies aim to mimic the periconceptual environment. As such, it is important to make sure they are optimised to the best of our knowledge. In the horse, there is a paucity of species-specific IVM optimisation. As for all inter-species comparisons, caution should be exercised in extrapolating from data from other species, such as the mouse or cow, for which there exists a wealth of knowledge on the subject (Leese, 2012). In this regard, the general aim of the work contained in this thesis was to characterise carbohydrate and oxidative metabolism in equine COCs during IVM, investigate it under varying clinical conditions, and ascertain their physiological relevance.

7.1 Summary findings

Below is a summary of the key findings from each chapter in context of the initial study aims and how the findings relate to current published literature.

Carbohydrate metabolism in equine COCs

The initial aim of these studies was to characterise the glucose, lactate, pyruvate and oxidative metabolism of equine COCs during IVM under commonly-used clinical conditions. It was demonstrated that COCs incubated in M199 based medium at 10- μ l medium per oocyte have high glucose consumption rates, utilise this glucose mainly for glycolysis, and also produce and secrete pyruvate into the medium (Figure 7.1). It was further shown that glucose consumption and lactate production were positively correlated to DNA content of the COC (and thus to number of cumulus cells present) but pyruvate production was not. COCs with compact cumulus (Cp) contained more DNA (more cumulus cells) and as such consumed more glucose than did COCs with expanded cumulus (Ex), however when glucose consumption was corrected for DNA content the difference disappeared. Additionally, glucose and lactate metabolism did not differ between COCs that were meiotically competent (matured to Metaphase II during IVM) or those that

degenerated during IVM, again suggesting that the main metabolic player in the COC is the cumulus, and indicating that cumulus function in these two cases was similar. These findings are in agreement with previous studies in the mouse, pig, cow and horse (Biggers et al., 1967; Downs et al., 2002; Gonzalez-Fernandez et al., 2018; Sutton et al., 2003). It is the first time however that data corrected for DNA (proxy for cumulus cell number) are reported for the horse.

Up to 24% of the COCs depleted all available glucose during the 30-hour IVM period in clinical conditions (10- μ l droplets of M199 based medium [5.6mM glucose]). Surprisingly, this did not appear to affect maturation, cleavage or blastocyst rates. Those COCs that had depleted all available glucose had a decreased glycolytic index (decreased lactate : glucose ratio) and decreased pyruvate production. This total glucose depletion has not been previously reported with regard to equine IVM. Adjustments of trimming excess granulosa and increasing the droplet size to 15 μ l (Chapter 4) reduced the proportion of COCs depleting all available glucose from 24% to 2.9%.

The studies in this thesis represent the first characterisation of COC oxidative metabolism together with mitochondrial function in any species. This was analysed in equine COCs using the Seahorse XFp analyser. Mitochondrial efficiency was evaluated at both the beginning and conclusion of IVM. There was a tendency for basal OCR to rise over the course of IVM but this was not statistically significant (mean 42.5 and 60.6 pmol O₂/ng DNA/ hour at the beginning and end of IVM respectively). The proportion of basal OCR coupled to ATP production (47-58%), non-mitochondrial processes (38-31%) and proton leak (17%) did not differ between the beginning and the end of IVM (Figure 7.1). Spare capacity however, changed significantly during IVM, starting at 85% and falling to 5% at the end. Basal OCR in the present study was ~20 times higher than that reported for bovine COCs under similar conditions (Sutton et al., 2003) demonstrating that OXPHOS has a more significant role in energy metabolism in the equine COC. There are no previous studies on mitochondrial function testing in COCs of any species to which to compare the present data, only a single bovine study and a single equine study

exist, both performed on denuded oocytes and showed that even at the denuded oocyte level, equine oocytes have a higher OCR than bovine oocytes (Obeidat et al., 2018; Sugimura et al., 2012).

Temporal differences in glucose, lactate and pyruvate metabolism in COCs during *in vitro* maturation were also evaluated for the first time. Glucose consumption increased during the middle period of IVM (10-20 hours) and fell again at the end (20-30 hours). Lactate production followed the same pattern. Pyruvate production however, was constant during the first 20 hours but increased sharply during the final period (Figure 7.1). Mural granulosa cells alone in culture did not exhibit this pattern, with constant glucose consumption and lactate production throughout the 30-hour period. Interestingly, isolated granulosa cells produced more pyruvate than did COCs at the outset of IVM but this subsequently decreased, and pyruvate production did not rise at the end of IVM as it did for COCs. The temporal pattern of glucose and lactate metabolism for equine COCs reported here differed from that in murine and bovine COCs. In these two species, glucose consumption increased over time but no corresponding increase in lactate production was detected such that the glycolytic index decreased (Preis et al., 2005; Sutton et al., 2003). In contrast, the equine COCs in the current studies maintained a constant lactate : glucose ratio throughout the culture period.

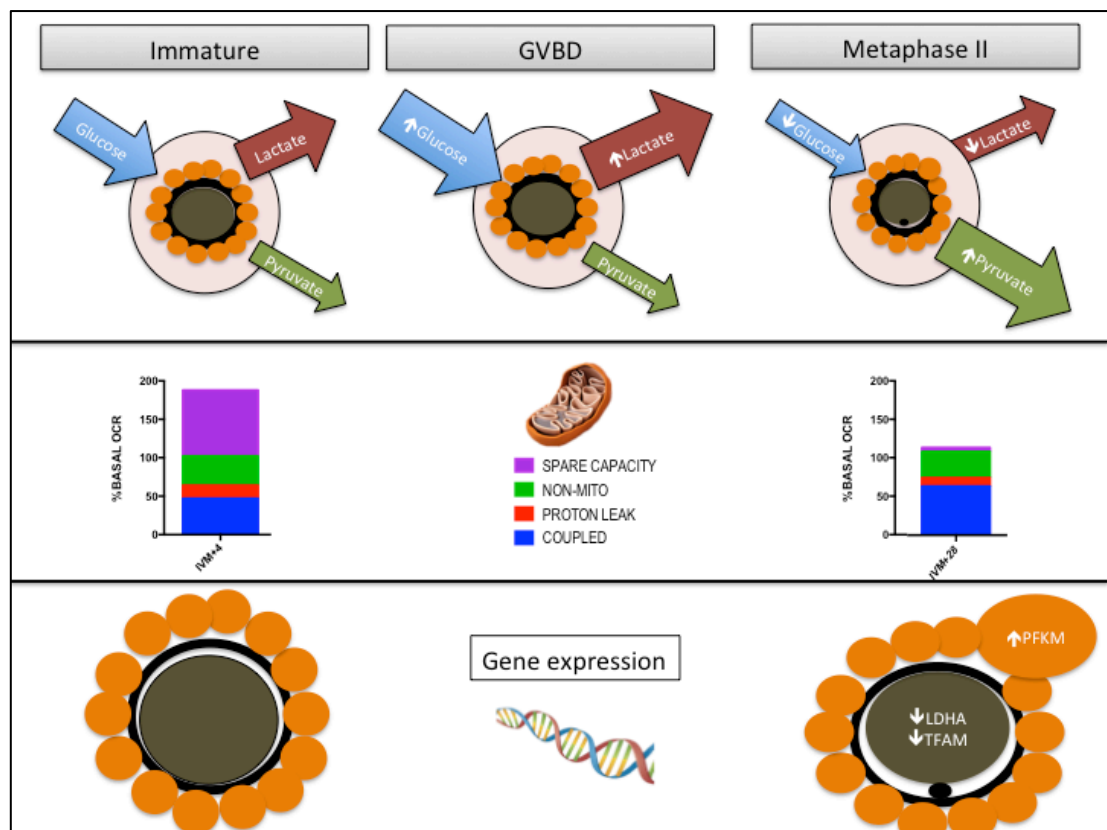


Figure 7.1: Summary figure of characterisation data from Chapters 3, 4, 5 and 6. Changes in glucose, lactate and pyruvate metabolism are depicted by arrows at the top. The middle section contains mitochondrial function data from the beginning and conclusion of IVM and the bottom section contains gene expression data of transcripts that differed between immature oocytes and metaphase II oocytes, arrows denote up or down regulation in either the oocyte or cumulus cell.

Pyruvate and oxygen concentration during equine IVM affect COC metabolism

In an attempt to address the glucose depletion and loss of spare capacity at the end of IVM and explore potentially more physiological IVM conditions for the horse, the addition of 0.15 mM pyruvate (similar to the concentration in equine follicular fluid) and a decrease in oxygen concentration (21% to 5%) was investigated. Paradoxically, there was an increase in glucose consumption in pyruvate-containing medium, independent of oxygen concentration, and at 5% oxygen in pyruvate-containing media, there was a reduction in pyruvate production and in glycolytic index compared to the other conditions (Figure 7.2). The effect of pyruvate concentration (up to 0.5 mM) on basal OCR and mitochondrial efficiency was also evaluated. There was a tendency for increased basal OCR at 0.5 mM pyruvate compared to 0 mM, however this was not statistically significant and pyruvate addition did not affect mitochondrial efficiency measures. The increase in glucose consumption in the presence of pyruvate and lack of response to decreased oxygen was unexpected. In Chapter 6, GPL measurements during IVM in medium with 0.5 mM pyruvate (M199 in the presence of 17mM and DMEM/F12) showed that COCs switched to net pyruvate consumption rather than production (Figure 7.2). To the best of my knowledge there are no other studies on direct evaluation of glucose, lactate and pyruvate metabolism in response to varying oxygen concentration during IVM; however COC gene expression data from other species suggest that, in contrast to our findings, glycolytic pathways are up regulated in the face of low oxygen (Bermejo-Álvarez et al., 2010, Hashimoto et al., 2000, Kang et al., 2011, Kumar et al., 2015).

Glucose concentration during IVM influences mitochondrial efficiency

In other species, IVM medium glucose concentration affects oocyte developmental competence (Frank et al., 2013, Hashimoto et al., 2000). In the horse, successful IVM and foal production is commonly reported using DMEM/F12, which, at 17 mM, is hyperglycaemic for the horse. The aim of these studies was to evaluate the effect of medium glucose concentration during IVM (5.6 mM vs. 17 mM) on oocyte developmental parameters and COC glucose, lactate, pyruvate and oxidative metabolism in an attempt to ascertain the consequences of hyperglycaemia during IVM at the metabolic level and understand the mechanisms by which equine COCs maintain their developmental potential in the face of hyperglycaemia.

Glucose concentration during IVM (5.6 vs. 17 mM) did not affect developmental parameters, morphokinetics of early embryo development, glucose consumption, and lactate/pyruvate production, or basal OCR. However, when evaluating mitochondrial function, it was found that IVM in 17 mM glucose resulted in a decrease in ATP-coupled respiration and an increase in non-mitochondrial respiration compared to that in 5.6 mM glucose (Figure 7.2). In other species studied, hyperglycaemia during oocyte maturation results in a decrease in glucose consumption attributed to inhibition of glucose transporters; thus the absence of an effect in equine COCs was surprising (Frank et al., 2014, Sutton-McDowall et al., 2010, Wang et al., 2010, 2012). While COC mitochondrial efficiency parameters have not been specifically investigated in other species in response to hyperglycaemia during IVM, other markers of mitochondrial function have been shown to be detrimentally affected. Namely, decreased cumulus cell ATP content and increased oocyte ROS production have been demonstrated in mice and cattle respectively, indicating mitochondrial effects of hyperglycaemia, in agreement with the current data (Hashimoto et al., 2000, Wang et al., 2010).

Glucose concentration during IVM and its effect on blastocyst gene expression

In order to investigate the potential downstream effects of glucose concentration during IVM (5.6 mM vs. 17 mM) on developmental potential, expression of genes coding carbohydrate-metabolism enzymes involved in key metabolic pathways known to be targets of hyperglycaemia were evaluated in resulting blastocysts. Developmental marker genes for pluripotency and cell lineage were also evaluated. Regrettably, blastocyst rates were too low (8.2 and 2.9% per injected oocyte for 5.6 mM and 17 mM, respectively) to satisfy the original experimental aim. However, the expression of glucose metabolism genes *SLC2A1*, *LDHA*, *G6PD*, *PFKM* and the TE marker *TEAD4* was described for the first time in equine blastocysts and further description of *POU5F1*, *SOX2* and *CDX2* added to limited knowledge of cell lineage allocation dynamics in the horse. To the best of my knowledge this was the first study to examine the downstream effects of glucose concentration during IVM on blastocyst gene expression in any species.

A secondary aim possible through the experimental design of single-oocyte tracking through maturation followed by mRNA extraction from blastocysts, was to explore associations between the relative expression of the above genes in the blastocysts and the corresponding COC metabolism during IVM. Use of time-lapse imaging during embryo culture allowed associations between the relative expression of the genes and blastocyst morphokinetics during embryo development to also be explored. Again, while some relationships were suggested, such as decreasing *LDHA* being associated with increasing *POU5F1* and *GPX1*, and increasing *GPX1* being associated with increasing *POU5F1* and *TEAD4*, overall blastocyst production was too low to satisfy the original aim and draw conclusions.

Glucose concentration during equine IVM and its effect on oocyte and cumulus cell gene expression

As noted above, increased glucose concentration during IVM resulted in altered mitochondrial function in the equine COC; however, an effect on blastocyst gene expression could not be determined. To give further insight into the effect of high glucose during IVM, cumulus cell and oocyte expression of metabolic genes was examined. DMEM/F12, one of the media used clinically for IVM in the horse, combines high glucose (17 mM) and pyruvate (0.5 mM). Due to the possibility that the pyruvate in DMEM/F12 ameliorates the effect of the high glucose, metabolic parameters in DMEM/F12 were compared not only to those for the other commonly-used IVM base medium, M199, but also those for: M199 medium with added glucose (17 mM) and M199 medium with added glucose and pyruvate (0.5 mM). In this way, it could be determined whether A) the increased glucose would alter metabolic parameters and B) the presence of pyruvate would ameliorate this effect. A significant effect of pyruvate was found: in cumulus cells, G6PD, an enzyme of the PPP pathway, was upregulated in M199 with 17 mM glucose and 0.5 mM pyruvate compared to M199 with 17 mM glucose alone. Unfortunately, biological variation was large and no differences were detected in gene expression attributable to experimental condition in oocytes.

Uniquely, the combination of metabolic evaluation and gene expression analysis allowed associations between relative gene expression in the oocyte/cumulus cell and COC glucose, lactate and pyruvate metabolism to be explored. In cumulus cells, expression of *GPX1*, was positively correlated to glucose consumption suggesting those COCs which were more metabolically active required a higher antioxidant capacity. In addition, expression of *TFAM*, was negatively correlated to lactate : glucose ratio (it increased as glycolysis proportionately decreased) and positively correlated to pyruvate production. Notably no relationship existed between the expression of genes in oocytes and COC metabolism, or between cumulus cell gene expression and oocyte gene expression.

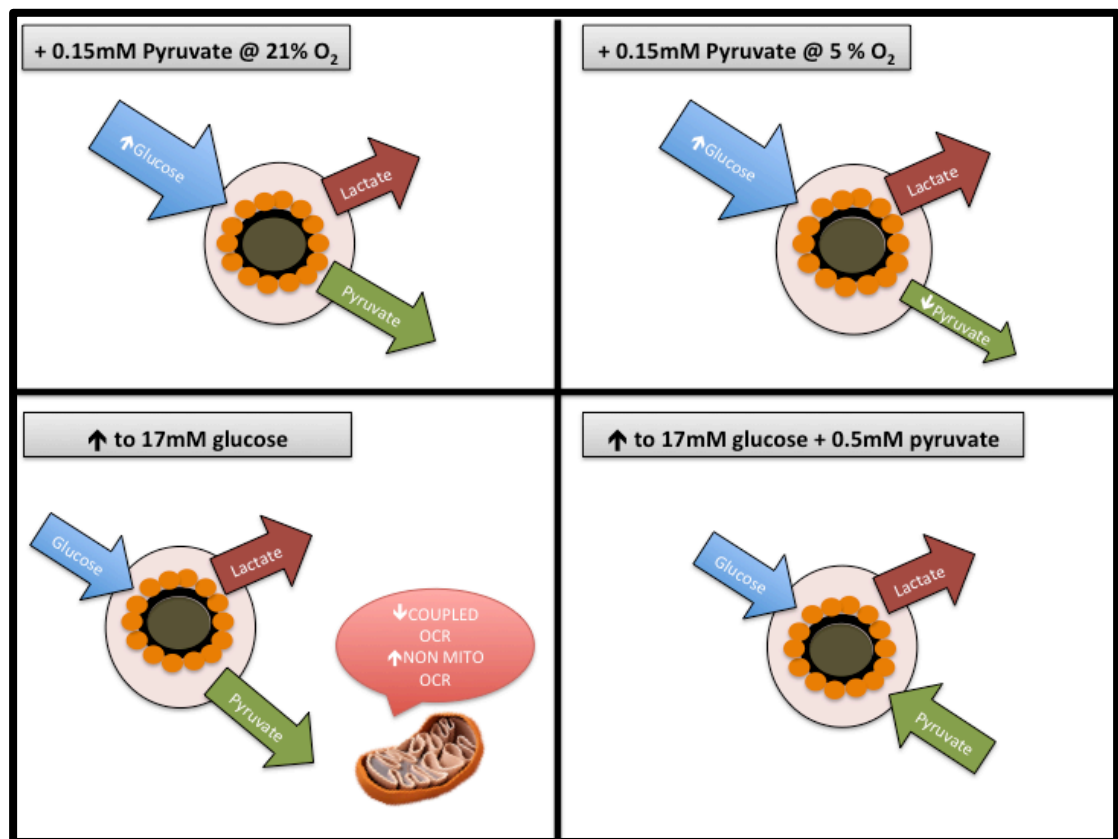


Figure 7.2: Summary diagram of significant findings from Chapters 4, 5 and 6 pertaining to medium adaptation. Glucose, lactate and pyruvate metabolism is depicted by arrows as increasing or decreasing compared to control conditions. Presence of 17 mM glucose resulted in changes in mitochondrial efficiency; decreased proportion of OCR coupled to ATP production and increase in the OCR attributable to non-mitochondrial processes.

7.2 Interpretive caution

The major caveat to interpreting data originating from abattoir-derived tissues is that of heterogeneity of animal age, breed and health status. In addition, as all follicles present on the ovary were used for oocyte recovery, there is great heterogeneity in the ovarian follicle population, even within the same animal. The scarcity of equine abattoirs and the difficulty in recovering meaningful numbers of oocytes from live animals in this species necessitates that inclusion criteria are lenient and as such a high biological variation is expected. Optimally, stringent inclusion criteria such as age and health status of mare, stage of follicle in relation to the follicular wave (i.e. status in terms of viability/atresia) and follicle size could reduce variation in results.

While this research represents pioneering work in evaluation of COC metabolism during IVM, some technical aspects of the experiments, such as performing OCR measurements on groups of COCs leave room for improvement. While the Seahorse XFp analyser had many benefits over existing methods to measure OCR, the relative low sensitivity necessitated equine COCs to be cultured in groups. As such, information pertaining to meiotic status or future developmental potential could not be assigned to individual oocytes.

7.3 Common themes, clinical implications and future work

What substrates are responsible for ATP production in the equine COC?

An estimate can be made of total ATP production by equine COCs by combining data from different experiments. Appearance and depletion of substrates can allow meaningful deductions. The production of one mole of lactate produces one mole ATP whereas approximately five moles of ATP are produced per mole of oxygen consumed ($2 \times \text{P/O max [O]}$ of 2.5; (Mookerjee et al., 2017). Across all current experiments lactate production was approximately 18 pmol/ng DNA/hour; this represents 18 pmol ATP. Considering OXPHOS, mean OCR was 46 pmol/ng DNA /hour of which 50% was coupled to ATP production, equating to 115 pmol ATP. Taken together it can be estimated that 13.5% of ATP production can be accounted for by glycolysis and 86.5% from OXPHOS i.e. the majority of ATP is being produced by OXPHOS which is in contrast to at least the bovine COC (Sutton et al., 2003). It is interesting to note, that at least from a functional perspective, stallion sperm are also different to other species in that they rely on OXPHOS and not glycolysis for both motility and membrane integrity (Davilla et al., 2016).

Now considering substrate use, the glycolytic index across all experiments (when glucose was not limiting) was close to 100 %. This suggests that glucose was not being directly oxidised and that another substrate(s) is involved in ATP production in the equine COC under clinical IVM conditions. The preferential conversion of glucose to lactate (i.e. favouring glycolysis), despite the presence of plentiful oxygen, is also a characteristic of cancer and other rapidly proliferating cells and is known as the Warburg effect (reviewed by Smith and Sturme, 2013). Developing embryos, particularly at the early blastocyst stage, are also known to convert most glucose consumed to lactate in the rat (Brison and Leese, 1994), mouse (Houghton et al., 1996) and cow (Thompson et al., 1996). Even at the lowest range of glycolytic index found in COCs in the presence of excess glucose in the current studies (lactate : glucose ratio 1.8), a maximum of 10 % of the glucose consumed would be available for oxidation. Since mean glucose consumption was 10 pmol/ng DNA/hour, 10% of

this would be 1 pmol of glucose oxidised. This would still only account for 30 pmol ATP representing only 26% of the total ATP from OXPHOS and indicating that a source other than glucose was responsible for the remaining 74% of the OXPHOS generated ATP.

The large proportion of OXPHOS derived ATP observed in the current study, is in stark contrast to data derived from bovine COCs under the same conditions (Sutton et al., 2003). In that study approx. 30-60 pmol ATP/ng DNA/hour could be attributed to lactate production and only ~5-17 pmol ATP/ng DNA/hour to OXPHOS representing just ~22% of overall ATP (vs. 86.5% in the equine COCs) and all of the OCR could be accounted for by pyruvate consumption. This finding, however, highlights the need to examine the composition of the experimental medium utilised; the study by Sutton et al was carried out in medium containing 0.23 mM pyruvate. In contrast, there was no added pyruvate in the basic equine maturation medium used in the present work. In this medium, the COCs preferentially produced and secreted pyruvate into the medium. Hence, the OCR in equine COCs in this medium cannot all be attributed to pyruvate. Speculating further, it is interesting that in M199 medium containing 0.5 mM pyruvate (in the presence of high glucose; 17mM- the amount present in DMEM/F12) COCs switched to net pyruvate consumption and the presence of pyruvate alone at 0.5 mM resulted in a non-significant increase in basal OCR. However, even with this amount of pyruvate consumption (~1 pmol/ng DNA/hour, equating to a maximum of 15 pmol ATP) total ATP production is still not accounted for. Therefore, the question remains- what substrates are equine COCs oxidising during IVM to produce the majority of their ATP? Furthermore, are these findings physiological in relation to what would occur *in vivo*?

The candidates for oxidation other than sugars are fatty acids, glycogen and amino acids. Glycogen stores have received little attention in the oocyte but are known to accumulate throughout the cleavage stages of embryo development, perhaps playing a more important role at the blastocyst stage (Ferguson and Leese, 1999, Houghton et al., 1996). Amino acid metabolism as a means of ATP generation has

not been widely studied, though it has been shown in COCs, oocytes and early embryos that there is significant turnover of amino acids from the culture medium and that the absence of certain amino acids can perturb development (Cetica et al., 2003; Gardner and Lane, 1993). Amino acid turnover in COCs and embryos is of interest as a potential bio-assay related to developmental potential (Hemmings et al., 2012; Picton et al., 2010). In support of their use as an oxidative substrate, it has been demonstrated that mouse oocytes promote amino acid transport by the cumulus cell (Eppig et al., 2005) and that transaminases, aspartate aminotransferase in particular, are highly active in the bovine oocyte suggestive of use of amino acids in the TCA cycle (Cetica et al., 2003). M199, being a complete culture medium provides all amino acids and investigating amino acid flux would be an interesting area of further study.

Despite amino acid and glycogen oxidation potentially contributing to ATP production, it is unlikely they could sustain such a high level of ATP production as reported here (Ferguson and Leese, 1999). Given the dark colour of the cytoplasm in the equine oocyte, similar to the pig, and the appearance of apparent lipid droplets on electron microscopy, it is assumed that the equine oocyte contains large amounts of lipid and by extrapolation, that fatty acid oxidation (FAO) has a major role in ATP generation in either the oocyte, the embryo or both. β -oxidation of fatty acids is known to be essential for nuclear maturation and optimal oocyte developmental competence in the mouse, cow and pig (Dunning et al., 2010; Paczkowski et al., 2013). Additionally, FAO provides greater amounts of ATP than does oxidation of an equivalent amount of glucose; as an example one mole of the fatty acid palmitate can produce 106 moles of ATP in contrast to the 30 that can be produced from one mole of glucose (Dunning and Robker, 2012). In the pig oocyte (incubated in TCM-199; 5.6 mM glucose), it was shown that intracellular triglyceride content reduced by 13 ng during the 44-hour maturation period confirming its utilisation as a metabolic substrate (Sturmey and Leese, 2003). IVM of bovine COCs in TCM-199 in the presence of methyl palmoxirate, an inhibitor of FAO, resulted in a 45% decrease in OCR of MII denuded oocytes further highlighting the relevance of FAO during IVM (Ferguson and Leese, 2006).

Taken together, the findings of these studies demonstrate the versatility in substrate use for ATP generation which characterises COCs, and appears to be influenced further by medium composition (Cetica et al., 2003). This is well-illustrated by the issue of pyruvate availability, consumption, and production. It would, for example, be very interesting to repeat the GPL and OCR measurements in COCs incubated in a wider range of pyruvate concentrations to identify if there is a preference for pyruvate oxidation if and when pyruvate is available and if so, the threshold pyruvate concentration at which this occurs. It would also be interesting to monitor triglyceride content, manipulate β -oxidation and observe the response in OCR.

In the murine embryo, there is some evidence to suggest that promoting the use of pyruvate as an oxidative substrate, through stimulating pyruvate dehydrogenase (PDH) activity with dichloroacetic acid (DCA), is beneficial in terms of embryo development (Mcpherson et al., 2014). PDH is also a therapeutic drug target in diabetic cardiomyopathy in which overutilisation of FA for oxidation leads to a decrease in myocardial function (Page et al., 2015) -demonstrating that versatility in substrate use is not always beneficial.

Is depletion of all available glucose during IVM unfavourable?

In the initial study (Chapter 3) it was discovered that a large proportion of COCs depleted all available glucose from 10- μ l droplets of M199 based medium containing 5.6 mM glucose. It was assumed that this suggested an inadequacy in the current clinical conditions and, importantly, it rendered the data from those COCs unusable for data collection, as they could not be analysed in terms of glycolytic index when glucose was a limiting factor. COCs which depleted all available glucose had, as would be expected, decreased lactate and pyruvate production (Figure 7.3). As the experimental design measured total flux, it is possible that production did not change during the period in which glucose was available for glycolysis, but that lactate and pyruvate were taken back up by the COC to act as oxidative substrates in the absence of glucose at the end of maturation. That presented a question- what was the impact of glucose depletion? If the COCs can compensate adequately by re-consuming secreted lactate and pyruvate perhaps there is no negative effect and rather the avid consumption of glucose shown by these COCs was a sentinel for quality? This hypothesis could be tested by comparing developmental competence, maturation, cleavage and blastocyst rates, which I did in Chapter 3. These parameters were seemingly unaffected by depletion of glucose by the COC during IVM; as 1/9 oocytes that depleted all glucose developed into a blastocyst (11%), an equivalent (numerically higher) rate to that for non-depleting oocytes (3/40, 7.5%). In Chapter 5, unfortunately blastocyst rates were too low to explore the effect of depletion on blastocyst gene expression. However analysis of the single blastocyst to form from a COC that had depleted all available glucose during IVM revealed it had the highest relative expression of *PFKM* and *LDHA*, was the only embryo to express *TFAM* and had the lowest *GPX1* expression. If confirmed in a larger sample size, it would be interesting to speculate that high rates of COC glucose metabolism programmed a high rate of glycolysis in the blastocyst as demonstrated by high *PFKM* and *LDHA* expression. Also interesting is the low *GPX1* expression given that high expression is associated with blastocyst quality in other species (Castillo-martín et al., 2014; Cebrian-Serrano et al., 2013). Furthermore, identical conditions in Chapter 6

allowed the effect of depletion on oocyte and cumulus gene expression to be explored. Two COCs in the control conditions (5.6 mM glucose, 0 mM pyruvate) depleted all available glucose. While the low numbers resulted in lack of significant differences, it was interesting that of the seven genes examined, *TFAM* was the only one for which both cumulus cell samples from depleting COCs expressed the gene above the mean and higher than all other samples. The implication of high expression of *TFAM* in both cumulus and the blastocyst in those COCs depleting all glucose is not clear. In most cell types high *TFAM* expression represents high quality but in cumulus cells from diabetic mice, *TFAM* expression is also elevated, presumably as a compensatory mechanism (Wang et al., 2010). *TFAM* expression in oocytes did not appear to differ between the two COCs that depleted glucose and those COCs that did not. In summary, while it seems that those COCs that deplete all available glucose have unique characteristics, it remains unclear if this is beneficial or not.

In considering the clinical equine IVM scenario, the ratio of maturation medium per COC varies and is often not stated; however, 10 μ l/COC is commonly used (Choi et al., 2016). One difference in clinical ICSI, is that oocytes are aspirated from follicles in live mares rather than scraped from follicles post mortem and as such contain many fewer cumulus cells than the full COCs in this experiment. However, work with slaughterhouse-derived COCs matured at 10 μ l medium per COC has resulted in high blastocyst rates (37-42%; Choi et al., 2007, Hinrichs et al., 2005, Ribeiro et al., 2008). A notable difference between these data and that of the current studies is that COCs are normally cultured in groups in research and clinical settings, in contrast to single culture in the present experiments. In this scenario, as only ~24% of COCs consume enough glucose to deplete it, the fact that the other 76% of COCs do not, may provide sufficient “spare” glucose that the group droplet is rarely depleted in practice. It is also possible that glucose metabolism differs between single and group maturation culture, meaning results cannot be directly extrapolated. However the results of Study 3.2 suggest that at least in groups of three COCs, glucose uptake per COC is similar to that for individual COCs. As total glucose depletion negated data use from that COC, adjustments were made in

Chapter 4 to prevent this. Excess granulosa trimming and increasing the droplet size to 15 μ l were effective in reducing the incidence of glucose depletion. I hypothesised that addition of pyruvate (0.15 mM) and lowering the oxygen concentration (5%) would further decrease glucose consumption, however unexpectedly, glucose consumption increased. Two further options to prevent total glucose depletion would be to refresh the media half way through IVM or provide more glucose. From a clinical standpoint, 17 mM glucose prevented the depletion of glucose, however, perhaps other strategies such as increasing the volume of the drop, trimming excess granulosa or refreshing the media are more physiological methods until the consequences of high glucose concentration are determined.

While I sought to examine the independent effect of 17 mM glucose in M199 medium based on the fact that clinical success has been reported using DMEM/F12 (17 mM), it cannot be ignored that DMEM/F12 differs markedly to M199 in more than just glucose concentration. It is possible that other differences in composition mean that glucose metabolism results obtained herein cannot be directly extrapolated. Importantly, DMEM/F12 also contains pyruvate (0.5 mM), a substrate not found in M199, which as shown in Chapter 6 resulted in pyruvate consumption rather than production. While glucose consumption and lactate/pyruvate production and gene expression was evaluated in COCs matured in DMEM/F12, OCR and mitochondrial efficiency was not. It would be interesting to examine basal OCR and mitochondrial efficiency in DMEM/F12 to determine if the additional components of this medium cause these parameters to differ from that seen in M199 with added glucose and pyruvate.

Based on the experimental data obtained in the current studies, my clinical recommendation would be to ensure tight trimming of excess granulosa and to increase the COC: medium ratio to at least 15 μ l to ensure depletion doesn't occur. This would prevent the COC from compensating at an as yet unknown cost.

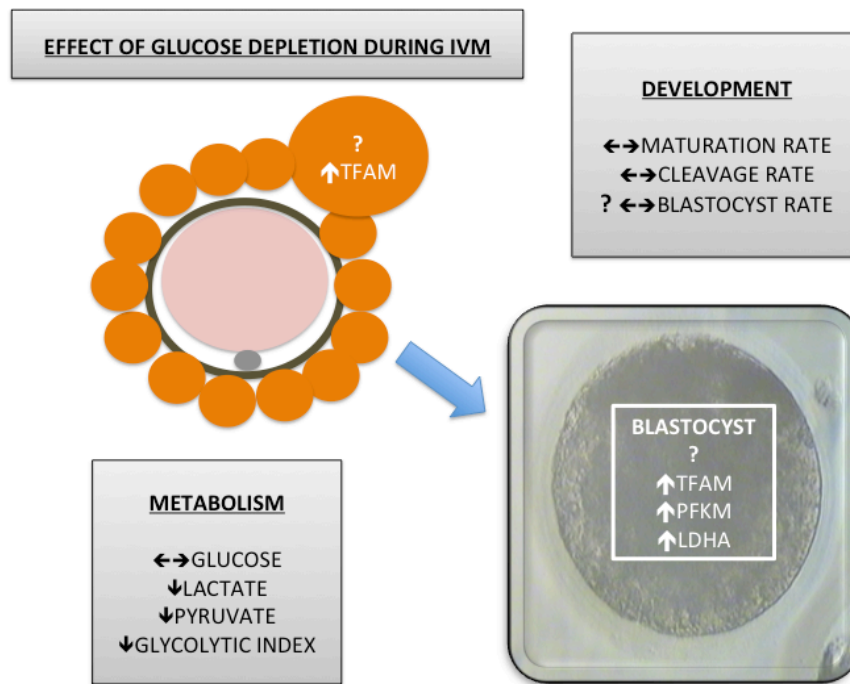


Figure 7.3: Schematic diagram summarising the effects of total glucose depletion by equine COCs during IVM on COC metabolic parameters, developmental markers and gene expression in cumulus cells and the resulting blastocyst. ? denotes suggested but unconfirmed observations.

Are the mitochondria of the COC compromised under current conditions?

Investigations into oocyte mitochondrial function and efficiency are very much centre stage in the assisted reproduction field, given the strong association between mitochondrial health and oocyte developmental competence (review; Van Blerkom, 2011). In fact, while somewhat controversial, procedures such as mitochondrial supplementation and nuclear spindle transfer are under development in human age-related infertility research and avoidance of mitochondrial disease transmission (Cagnone et al., 2016; Hyslop et al., 2016; Zhang et al., 2017). Examining mitochondrial efficiency in the experiments contained in this thesis consistently revealed a lack of spare capacity by the conclusion of IVM,

independent of medium. As experiments were performed on whole COCs, it is unclear what proportion can be attributed to cumulus cells and what to the oocyte. While of course there are many more cumulus cells, the oocyte contains up to 100-fold the number of mitochondria than does one cumulus cell. If cumulus cells are mostly represented, as speculated in Chapter 3 (equine denuded oocyte OCR data represented 10% of that measured in whole COCs in the present study [Obeidat et al., 2018]), it is possible that the lack of spare capacity is evolutionary in that cumulus cells are nearing the end of their physiological role and lifespan. However, the relative quiescence of each mitochondrial vestment is unknown and if a significant proportion of the oocyte mitochondria are represented, then lack of spare capacity and a high non-mitochondrial component suggests inefficiency. Given the role of oocyte mitochondrial dysfunction in aneuploidy and also the absolute requirement for a highly polarised mitochondrial membrane to sustain calcium oscillations during fertilisation (Dumollard et al., 2004) it is of great clinical importance to discover if oocyte mitochondria are indeed compromised in the current IVM conditions.

The high non-mitochondrial component of OCR, particularly in high glucose medium, suggests excess ROS production, as summarised in Chapter 5. As such it was notable that *GPX1*, an anti-oxidizing agent, was the gene with the highest expression in all three sample types (cumulus cells, oocytes and blastocysts). It could be speculated that this was in response to oxidative stress and further work should include direct measurement of ROS. It is worthy of mention that the three studies available examining the effect of antioxidant addition to equine IVM medium did not report a positive effect on developmental parameters or measures of oxidative stress (Deleuze et al., 2010; Luciano et al., 2006; Merlo et al., 2016). However, these results require interpretation in context of the medium in which the experiments were performed. The two studies investigating cysteine addition were carried out in M199 (5.6 mM glucose) and the third, using β -mercaptoethanol was carried out in DMEM/F12 (17 mM). The addition of antioxidants is worthy of further investigation.

COC metabolism and developmental markers

At the outset of these studies it was expected that a higher blastocyst rate would be obtained, based on success with this technique experienced during the preliminary studies (14% blastocyst rate). This would enable evaluation of associations between glucose, lactate and pyruvate metabolism and developmental competence. This was not fully possible, due the low blastocyst rate achieved during the studies. Unfortunately, the factors affecting variation in blastocyst production in the horse have not yet been elucidated, and such fluctuation appears to be a common problem, as can be seen by varying rates reported even within established equine ICSI laboratories. However it was interesting to note that the COC glucose consumption and lactate production during IVM of those oocytes that became blastocysts was close to the exact mean of all COCs. While numbers are obviously low, this pattern is in agreement with the ‘Dynamic Energy Budget Theory’ proposed by Professor Bas Kooijman (2010) and demonstrated in bovine embryos by Guerif et al., (2013). A recent review has termed this the “Goldilocks principle” whereby embryos with maximal developmental potential have a metabolic rate at “just the right amount” (Leese et al., 2016). In contrast, in studies examining COC metabolism in relation to blastocyst development in the cow, mouse and cat, high glucose consumption was associated with improved blastocyst development (Krisher and Bavister, 1999; Preis et al., 2005; Spindler et al., 2000).

In considering possible developmental markers from the gene expression data in Chapter 5, *POU5F1* tended to be associated with both speed of development and *GPX1* expression. Given that both *POU5F1* and *GPX1* have been suggested as developmental markers of competence in other species (Castillo-martín et al., 2014; Cebrian-Serrano et al., 2013; Filliers et al., 2012), they warrant further study in the horse.

Investigation of the legacy effect of equine IVM culture conditions is required

The downstream consequences of suboptimal IVM conditions are as yet relatively undefined, particularly in the horse. While I attempted to address this by evaluating blastocyst gene expression, the aim could not be fulfilled. However, I still believe this is a crucial aspect of determining IVM condition suitability. It is clear that good blastocyst rates and foal production can occur under the current conditions and anecdotal evidence exists of excellent performance in the resulting adult horses. This indicates that the equine COC is able to adapt to culture conditions, however, as reviewed by Roseboom, (2018) this adaptation can occur at the detriment of long-term health outcomes, indices which have not yet been examined for foals produced by equine ART. Ideally, this would involve phenotyping foals produced from varying conditions. A recent study by Valenzuela et al., (2017) investigating the effects of equine assisted reproduction techniques (embryo transfer and ICSI) on foal and placenta morphometry and expression of selected placental genes, interestingly found no significant differences. However, a study exploring the effect of mare breed and uterine environment demonstrated an influence at the metabolic level in foals (Peugnet et al., 2014).

The mechanism by which gamete or embryo environmental variation results in a heritable change of phenotype is epigenetics, that is, the intracellular control of gene expression. Epigenetics is at the centre of the Developmental Origins of Health and Disease (DOHAD) concept, which was first described by David Barker. The DOHAD concept is currently considered to be of immense importance given the worldwide epidemic of non-communicable diseases in humans, the risk for many of which has now been linked to the periconception and gestational period (recently reviewed by Fleming et al., 2018). Thus, evaluating the epigenetic effects of varying IVM conditions is a further approach that could be employed to decipher suitability. In this regard, aberrations in gene methylation, decreased expression of DNA methyl transferases (DNMTs) and altered histone acetylation have been demonstrated in oocytes derived from diabetic mice (Ding et al., 2012; Ge et al., 2014, 2013; Ma et al., 2012). To the best of my knowledge there are only two

studies (performed by the same group) investigating epigenetic effects in equine oocytes. The first study performed by Franciosi et al., (2012) identified global histone 4 deacetylation on lysine residue 16 (H4K16) in *IVM* oocytes where as H4K16 was acetylated in *in vivo* matured oocytes. As histone acetylation is a critical epigenetic regulator of processes such as chromatin condensation, these authors hypothesised that the observed deacetylation was responsible for the increased aneuploidy rates and spindle defects they observed in the *IVM* oocytes compared to *in vivo* matured oocytes (Franciosi et al., 2015). While this association remains unconfirmed, this and other epigenetic mechanisms are certainly areas that could be explored further in the context of the experiments described in this thesis.

Novel experimental designs and proof of concept

A notable feature of the work described in this thesis is that for many of the metabolic methods utilised, this is the first time they have been applied to COCs of any species. Enzyme-linked assays, oxygen consumption measurements using the Seahorse XFp analyser, and PolyA PCR amplification all required development and optimisation. Additionally, novel experimental designs enabled ambitious aims such as exploring associations between oocyte, cumulus and blastocyst gene expression and COC metabolism and morphokinetics of embryo development to be investigated. Unfortunately, inadequate numbers resulted in these aims not being fully addressed, however proof of principle was demonstrated and the value of the experimental design highlighted. Further uses for future research would include banking media from clinical cases in which blastocysts produced are ultimately destined for transfer. GPL measurements and cumulus gene expression could then be performed without sacrifice of the oocyte and potentially allow for more detailed investigations. If the Seahorse XFp analyser sensitivity could be enhanced, measuring OCR of single COCs and/or embryos would also allow further interpretation of current data.

7.4 Concluding remarks

Data reported in this thesis characterised for the first time the carbohydrate energy metabolism of the equine COC during IVM, and began to explore the suitability of commonly used IVM medium and metabolic substrates for equine IVM, with reference to both mitochondrial function and developmental parameters. Additionally, I have contributed to existing data on cumulus, oocyte and blastocyst gene expression and have validated exciting platforms for future studies.

I believe that data produced and literature reviewed in the course of this thesis demonstrates not only the scientific value of exploring the equine COC and embryo to increase our knowledge of the comparative biology of these fundamental phases of mammalian life, but also the importance of continuing this work to ascertain the suitability of current equine IVM media and conditions used for clinical foal production. A recommendation can be made at the very least to exclude elements known to be non-physiological (e.g. 17-mM glucose during IVM) until such a point that conclusive evidence exists that they do no harm.

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Appendix 1: Published paper containing material from Section 1.5

Lewis N.L. and Sturmev R.G.S. 2015. Embryo Metabolism: What does it really mean?
Anim. Reprod. 12, 521-528

Embryo metabolism: what does it really mean?

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Abstract

The study of early embryo metabolism has fascinated researchers in the field for nearly a century. Herein, we give a brief account of the general features of embryo metabolism and some consideration of the research performed to reach such conclusions. It is becoming increasingly obvious that metabolism informs many fate decisions and outcomes beyond ATP generation, such as DNA methylation, Reactive Oxygen Species generation and cell signaling. We discuss the reasons for studying metabolism in the face of our current knowledge of the effect that the culture environment on the developing embryo and the downstream effects that can cause. The study of *in vitro* embryo metabolism can also give us insight into developmental perturbations *in vivo*. The strengths and limitations of the methods we use to study metabolism are reviewed with reference to species-specific fundamental biology and plasticity and we discuss what the future holds for metabolic studies and the unanswered questions that remain.

Keywords: ATP generation, method evaluation, preimplantation development.

Introduction

The study of mammalian early embryo metabolism has a rich history (Leese, 2012). Whilst work in the period of the 1940s-1960s focused on the effect of adding energy substrates to embryos in culture, real progress in understanding embryo metabolism was made in the 1970s by the likes of Biggers and Stern (1973), Brinster (1973) and Gwatkin and Haidri (1974) who examined the fate of radiolabeled compounds added to the medium. From experiments such as these, a picture of early embryo metabolism began to emerge. Like so much of our knowledge of early mammalian embryo development, the first data came from the classical laboratory model species; mouse and rabbit, as well as the hamster. Interest grew, and embryo metabolism was soon examined in the large domestic animals; pigs, cattle, sheep and, to a lesser extent, the horse, dog and cat. Underpinning research were studies on early human development with the aim of clinical translation for the treatment of infertility; a feat first achieved in 1978 by Steptoe and Edwards. Alongside

this feat was the development of assisted conception techniques for use in farm animals. It is not the intention of this article to re-describe the history of the research that led to successful embryo culture or the contribution that studies on metabolism made. For expert insight, the reader is encouraged to read (Leese, 2012; Chronopoulou and Harper, 2014).

Embryo metabolism: what do we know?

The description of carbohydrate metabolism during preimplantation development is largely accepted and will be familiar to anyone who has an interest in the early embryo. In almost all species studied, the cleavage stage embryo, from fertilisation through to formation of the morula, is relatively metabolically quiescent. Oxygen consumption at this time remains comparatively low, and the dominant substrate depleted from the culture environment is pyruvate. Pyruvate is consumed at an almost steady rate during cleavage, with a proportion of the carbon (depending on the species) appearing in the medium as lactate with the generation of metabolic energy. The source of the pyruvate involved in such reactions is generally either glycolytic conversion of glucose or that taken up directly from the external environment. Pyruvate may also enter the Tricarboxylic Acid (TCA; Krebs) cycle, where it can be oxidised completely generating electron donors for the electron transport chain which occurs in the matrix of mitochondria and relies on oxygen acting as the terminal electron acceptor. For this reason, oxygen consumption provides a good marker of overall oxidative metabolic activity (for review, see Smith and Sturme, 2013).

As the cleavage stage embryo progresses to a blastocyst, there is a sharp and characteristic rise in the amount of glucose consumed in all species studied, and a concomitant rise in lactate release into the medium. Coincident with this is a fall in pyruvate consumption. This general pattern of “blastocyst glycolysis” appears to be conserved across all species studied. There are a range of explanations for this, however, as glycolysis is a comparatively inefficient means of generating ATP therefore energy production is unlikely to be the prime reason. Moreover, as the blastocyst forms, oxygen consumption also rises (Fridhandler *et al.*, 1957; Houghton *et al.*, 1996; Thompson *et al.*, 1996; Trimarchi *et al.*, 2000; Sturme and Leese, 2003)

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further supporting the notion that glycolytic production of lactate is of minor consequence in contributing ATP for the blastocyst. It is much more likely that glycolysis rises to meet the need for carbon for biosynthetic processes. A description of glycolysis in the early embryo can be found in Smith and Sturme (2013). This general picture of embryo metabolism was summarized with great prescience by Brinster in 1973; in the intervening years many laboratories across the world have generated evidence to support such a description, illustrating the robustness with which these findings can be considered.

While early work focused on carbohydrate metabolism, it is now clear that the metabolism of amino acids, lipids and vitamins such as folate all also act in an interdependent manner to produce a viable embryo. Amino acids are crucial components of the culture environment *in vitro* (reviewed by Sturme *et al.*, 2010). Their addition to simple culture medium either singly (Rieger *et al.*, 1992a) or in combinations (Chatot *et al.*, 1989; Gardner and Lane, 1993) permitted mouse embryos to be cultured past the so-called 2-cell block (Chatot *et al.*, 1989) and their widespread inclusion lead to improved blastocyst rates in almost every species studied. The addition of amino acids has had such a positive effect on the efficacy of *in vitro* embryo culture, that their inclusion is often described as having a primary role in the formulation of “next generation medium” (Leese, 2012). The precise mechanism for the positive effect of amino acid provision is still to be defined, however it is well established that addition of amino acids to *in vitro* medium can alleviate culture associated stress in flushed murine embryos (Lane and Gardner, 1998). The contribution that amino acid metabolism makes to ATP production remains unclear, however the turnover of amino acids (that is, the sum of their depletion or accumulation into the culture droplet) has been linked to embryo blastocyst rates (Houghton *et al.*, 2002), human embryo live birth rates (Brison *et al.*, 2004), DNA damage (Sturme, 2009), aneuploidy (Picton *et al.*, 2010) embryo sex (Sturme *et al.*, 2009a), maternal age (Picton *et al.*, 2010) and embryonic stress (Wale and Gardner, 2012).

When considering energy metabolism of early embryos, it is vital that the contribution made by endogenous triglyceride is not overlooked. Fatty acid β -oxidation was studied in detail in the 1970s by Kane and colleagues (Kane, 1979) but then largely ignored, with the notable exception of the work by Downs (see Downs, 2015). However, interest in fatty acid metabolism has re-awakened, partly in response to the report from Dunning *et al.* (2010) who elegantly demonstrated that mouse oocytes require fatty acid oxidation in order to develop. A similar conclusion was drawn by Sturme and Leese (2003) in the pig, underlining the importance of fatty acid β -oxidation during oocyte maturation, development and in the

preimplantation stages. Species differences in the importance of fatty acid oxidation during oocyte and embryo development have also been identified. For example, where a mouse zygote will arrest after 15 h in media lacking nutrients (cited in Leese, 2012) a rabbit embryo can complete up to 3 cleavage divisions in the absence of energy substrates (Kane, 1987) and sheep embryos can also develop to the blastocyst stage in the absence of glucose (Thompson *et al.*, 1992). This can be explained by the differences in intracellular triglyceride content, acting in a buffering capacity by providing an alternate energy source (Ferguson and Leese, 2006; Sturme *et al.*, 2009b). Recently, a number of laboratories have described altered fatty acid metabolism by embryos from overweight and obese mice (Pantasri *et al.*, 2015; Reynolds *et al.*, 2015) and the human (Leary *et al.*, 2014). After receiving comparatively little attention since the work of Kane, interest in fatty acid metabolism by oocytes and embryos has been intense, and has been widely reviewed in recent years (Sturme *et al.*, 2009b; Leroy *et al.*, 2012; McKeegan and Sturme, 2011; Dunning *et al.*, 2014; Downs, 2015).

This very brief overview is intended to remind the reader of the basic features of early embryo energy metabolism. However, ‘metabolism’ refers to significantly more functions than ATP generation. For example, there is an extensive literature describing the role of the pentose phosphate pathway (Downs *et al.*, 1998; Sutton-McDowall *et al.*, 2010) in mammalian oocytes and early embryos. Moreover, metabolic processes link to signaling mechanisms (Manser and Houghton, 2006), generation of Reactive Oxygen Species (Agarwal *et al.*, 2005) and gene expression in terms of establishment of epigenetic marks such as methylation and acetylation and post-translational modifications of proteins (DeBerardinis and Thompson, 2012). For example, defects in folate metabolism have been linked to methylation and epigenetic modifications affecting developmental competence (Xu and Sinclair, 2015). However, reviewing all of the literature on embryo metabolism in its broadest sense would require several articles and so in the remainder of this article, we will consider some more fundamental aspects.

Why do we study embryo metabolism?

Understanding the basic physiology and metabolism of the early embryo is a noble quest in itself that has fascinated researchers over the past decades. However, a major gap in our knowledge is the metabolism of the *in vivo* produced embryo, as well as the embryo *in situ*, which remain an elusive goal. We aim to gain information that can, and has been, translated into clinical practice in many ways; to design appropriate species specific culture media with the aim of producing viable healthy offspring; to design non-invasive methods for embryo selection for transfer and

shed light on metabolic perturbations occurring *in vivo*. Moreover, as our understanding of somatic cell nuclear transfer (SCNT; Wilmut *et al.*, 2002) grows and becomes linked inextricably to stem cell physiology and regenerative medicine, we must also accept that we know comparatively little about the impact of such techniques may have on embryo physiology. Furthermore, we are on the brink of many new and exciting developments in Assisted Conception, including mitochondrial transfer for the treatment of debilitating hereditary conditions as well as the replenishment of mitochondria in aged oocytes with the aim of improving pregnancy rates in older women (Craven *et al.*, 2010; Smeets, 2013). Such techniques may be considered ‘beyond experimental’; mitochondrial transfer was licensed for treatment in the UK in 2014 and autologous mitochondrial transfer for infertility is already commercially available in some countries. However, since each of the approaches described above involve, in some way, altering the mitochondrial content of embryos, the need for detailed understanding of metabolic regulation of individual preimplantation mammalian embryo has never been greater.

A further drive to study embryo metabolism comes from the need to identify biomarkers of embryo health and viability. This relies on the inherent variability in metabolism between different embryos and has been used in an attempt to select viable embryos for transfer, with the end goal being clinical IVF in humans. There have been several observations that have yielded promising results. The ‘quiet embryo hypothesis’ proposed by Leese in 2002, stated that those embryos that are viable have a decreased metabolic rate; a proposition that has been supported by several studies showing embryos with an upregulated metabolism of both carbohydrates and amino acids to have decreased viability post transfer (Lane and Gardner, 1996; Sturme *et al.*, 2009a; Guerif *et al.*, 2013). However, the notion is contested, and there are recent studies suggesting that elevated metabolism, particularly with respect to glucose consumption is associated with embryo viability (Gardner *et al.*, 2011). Clearly, this is an area in which more work is needed.

Since pioneering observations linking human birth weight to cardiovascular events in later life by David Barker *et al.* (1989) it has now been shown unequivocally in many species that the periconceptual environment can have downstream effects which can impact on the viability of the developing embryo and on the future health of the resulting offspring (Ceelen *et al.*, 2008; Watkins *et al.*, 2008; Leroy *et al.*, 2009; Fleming *et al.*, 2012; Frank *et al.*, 2014). It is also clear that certain embryonic stages are more susceptible to damage (Rieger *et al.*, 1992a), such as the early cleavage embryo during embryonic genome activation, suggesting that progeny may have a ‘memory’ of their origins.

With the rising obesity epidemic both in humans and companion animals, in addition to metabolic disease in farm animal species due to increased production pressures, the study of embryo metabolism *in vitro* can provide insight into the mechanisms of resultant suppressed fertility and potentially identify therapeutic interventions.

These are important reasons for studying embryo metabolism, and it is clear that metabolic processes can directly influence gene expression (Van Hoeck *et al.*, 2011, 2013), and patterning of the embryo (Leary *et al.*, 2014). However, it is also of fundamental importance to be aware of what is measured when studying embryo metabolism. In the final part of this review, we will describe the strengths and limitations of embryo metabolic studies.

What are we actually measuring?

The measurement of embryo metabolism is faced with many technical challenges. Critically, the *in vivo* environment is still largely unknown for most species, meaning that the extrapolation of knowledge to an embryo *in vivo* is of questionable validity. The data available on embryo metabolism inform us of the strategy of substrate depletion and appearance in a given milieu. *In vitro*, this milieu is constrained by the addition of a limited number of substrates at static levels; supply and ratio of substrates varies only in response to an embryo’s own activity. This is in stark contrast to the situation *in vivo*, which is dynamic and responsive (Leese *et al.*, 2008). Even in species for which the *in vivo* embryo environment has been described, the method used to define it should be noted. Often *post mortem* changes and/or inflammatory changes due to catheterization can influence results thus making samples non-representative (Leese *et al.*, 2008). Moreover, the embryo *in situ* likely exists in a microenvironment within the oviduct, thus any subtle, specific composition features will be lost in flushing of the tube.

Given the heterogeneity in developmental potential, measures pertaining to single embryos are key and thus highly sensitive assays are needed. Both the use of radiolabelled substrates (Rieger *et al.*, 1992b) and enzyme-linked fluorescence assays to detect the appearance and disappearance of a substrate from culture media have been described (Leese and Barton, 1984; Guerif *et al.*, 2013). The relative metabolic quiescence of single embryos means that ‘analysis media’ (that is a medium in which the concentrations of substrates is reduced to enable measurement of change) is often used in order to permit detection of changes in substrate concentration (Hardy *et al.*, 1989; Sturme and Leese, 2003). This ‘analysis medium’ is often different to the *in vitro* culture media known to support development for most species, which, in turn differs vastly to the *in vivo* environment. Of course, it also must

be realized that there are many complex cell transport and metabolic pathways involved, and notions of influx and efflux leads' us to make what are essentially educated guesses about what occurs in the cell. Despite these limitations, these assays have greatly advanced our knowledge of metabolic pathways involved and have yielded highly repeatable results across different laboratories. Further methods that have been used to detect metabolic activity of embryos include culturing individually in micro-droplets or in large groups of embryos. However, the resolution of data from group culture is reduced since individual embryo heterogeneity is lost by 'averaging'.

New promising studies using NMR metabolomic technology, where substrate flux can be measured *in situ* have been recently described (Krisher *et al.*, 2015), however the subsequent interpretation and analysis of the complex data acquired presents new challenges.

Inferences about the contribution of oxidative metabolism are usually derived from measuring oxygen consumption. Methods vary, the most widely used being pyrene fluorescence (Houghton *et al.*, 1996) and nanorespirometry (Lopes *et al.*, 2010). Again while allowing accurate measurement of oxygen depletion in single embryos and seemingly not affecting development (Lopes *et al.*, 2005), the methods represents a significant 'alien' environment for the embryo.

Studies involving metabolic inhibitors and enzymatic co-factors have also added to our knowledge of embryo metabolism and in some cases provided the initial proof of certain pathways occurring and either being essential or non-essential for development. Among these, Brison and Leese (1994) showed that oxidative phosphorylation was not an absolute requirement for blastocoele formation in the rat by culturing embryos in the presence of cyanide, while Macháty *et al.* (2001) indicated that suppression of oxidative phosphorylation at the morula stage improved development to the blastocyst in the pig. Moreover, Dunning *et al.* (2010) have shown that β -oxidation is essential for optimal development in the mouse by culturing in the presence of etomoxir. In some cases, inhibition of certain metabolic pathways has been shown to improve developmental potential; for example the addition of EDTA to embryo culture medium (Gardner *et al.*, 2000). Although the mechanism is not confirmed, one possible role of EDTA in embryo culture medium is the suppression of glycolysis (Gardner *et al.*, 2000). However, it is equally likely that EDTA acts as an antioxidant by sequestration of metal ions which would otherwise catalyse the formation of Reactive Oxygen Species (Orsi and Leese, 2001). Studies such as these illustrate the importance of appropriate regulation of metabolic pathways during development and also indicate why it is necessary for pathways to be correctly orchestrated to match needs at

a given stage of development.

It all depends on the environment

It could be argued that measuring embryo metabolism *in vitro* (by necessity) amounts to measuring a stress response. This issue must be considered given the extremely adaptable nature of embryos of all species. Metabolism is necessarily dynamic, enabling rapid changes in needs to be met to maintain development. However, such dynamism means that the metabolic profile of an embryo can respond quickly in response to a change in external environment, shown clearly in mice, where perturbations occur within 3 h of *in vitro* culture in flushed *in vivo* blastocysts (Lane and Gardner, 1998). Both the presence and relative quantities of metabolic substrates in the environment in which experiments are conducted will significantly affect the results. While not attempting to provide a detailed discussion on the controversial aspects of *in vitro* culture systems, which still vary widely across laboratories, this point can be further illustrated by the differential metabolism that results from the presence or absence of serum and the atmospheric oxygen concentration (Wale and Gardner, 2010).

While the human IVF industry has moved towards defined culture media using macromolecular sources such as recombinant albumin, serum is still used in many production animal systems. Culture with serum has been shown to increase blastocyst development rates in the horse (Choi *et al.*, 2004) and the kinetics of blastocyst development in the cow (Rizos *et al.*, 2003). However, its presence has also been associated with increased intracellular lipid content (Ferguson and Leese, 2006) and altered metabolism (Reis *et al.*, 2003), up-regulation of oxidative stress and inflammatory pathways (Cagnone and Sirard, 2014) and decreased survival after vitrification (Gómez *et al.*, 2008). In addition, the oxygen tension of the reproductive tract in all species studied has been found to be below 10% (Fischer and Bavister, 1993). In terms of the environmental gas profile, there is now unequivocal evidence to support the notion that 20% oxygen reduces embryo development (Thompson *et al.*, 1990; Wale and Gardner, 2010) and that culture in low oxygen (5%) results in metabolic and proteomic profiles more closely matching *in vivo* counterparts (Thompson *et al.*, 1990; Katz-Jaffe *et al.*, 2005). Clearly, these factors will influence the results of any metabolic study and must be kept in mind when comparing studies.

In addition to the embryo adapting to its environment, the culture environment itself is not static. Depletion and accumulation of excreted substrates such as lactate and amino acids will change the local environment. Spontaneous de-amination will occur at 37°C, especially of glutamine, resulting in ammonium build up (Gardner and Lane, 1993), lactate build up may

overwhelm pH buffering system of the media and depletion of energy substrates can lead to alternative ATP generating pathways being used (Kane, 1987).

It is also important to note that the manner in which an embryo responds to its environment is species specific. This can be seen in differences in response to hyperglycaemia. While species such as rodents and humans, will have significant diminished development in the presence of high glucose (Moley *et al.*, 1998; Frank *et al.*, 2014), others such as the horse and pig are apparently unaffected (Sturme and Leese, 2003; Choi *et al.*, 2015). Qualitative testing of equine embryos produced in hyperglycaemic conditions however, highlights subtle differences not reflected in the blastocyst development rate such as a decrease in ICM cell number allocation (also observed in the rat) and known to be mediated through apoptosis (Moley *et al.*, 1998; Choi *et al.*, 2015).

It is thus vital to consider that studies on embryo metabolism provide us a snapshot of physiology in a given set of conditions. Whilst such data are of fundamental importance, care must be taken when extrapolating and comparing such information. It is thus much more desirable that studies on the depletion and appearance of embryo metabolism are reinforced by consideration of mechanisms of metabolic regulation of early development.

Embryo metabolism: some unanswered questions

As the emphasis in human IVF is increasingly on single embryo transfer, the identification of reliable non-invasive methods of determining embryo quality to maximize pregnancy rate per transfer remains the Holy Grail. Moreover, in species such as the horse where *in vitro* embryo production is rapidly generating interest, a specific tailored culture media has yet to be formulated. Whilst acceptable blastocyst rates (41%) and pregnancy rates after transfer (66%) can be achieved by some laboratories in the horse using cell culture media such as DMEM-F12, (Jacobson *et al.*, 2010; Hinrichs *et al.*, 2014) the more subtle effects of potentially inappropriate culture conditions leading to decreased viability remain to be seen. Identifying optimal species-specific culture systems presents an exciting challenge for those involved in studying embryo metabolism.

Sex selection is another lively area of embryo metabolism. Ethical considerations preclude the implementation of sex selection in the human, but in the production animal industry, and in dairy cattle in particular, appropriate non-invasive identification of sex before transfer would be an application with many uses. Promising results have been presented so far showing that both glucose metabolism and amino acid metabolism varies with sex (Sturme *et al.*, 2010; for review see Gardner *et al.*, 2010), however more work will need performed to increase specificity in order for the technology to make the transition to commercial practice.

New information is emerging all the time on the far-reaching downstream effects of aberrations in early embryo metabolism (Harrison and Langley-Evans, 2009). Given the clear links between the periconceptual environment and sub-optimal health outcomes in the human (Barker *et al.*, 2002) and production species such as the bovine (for example, the so-called Large Offspring Syndrome; Young *et al.*, 1998), understanding and attempting to mitigate the negative effects on suboptimal embryo development and life-long health of the offspring is an important area for future study (Leese, 2014).

Conclusions

It is acknowledged “that metabolism pervades every aspect of cell physiology” (DeBerardinis and Thompson, 2012) and this is especially pertinent to the developmentally plastic early mammalian embryo. As genomic, transcriptomic and imaging techniques advance we will be able to expand our understanding of embryo metabolism and how it links inextricably with developmental pathways through subsequent stages of gestation leading to the birth of a healthy offspring. It is the responsibility of us all working in the earliest stages of this process to understand the periconceptual environmental challenges faced by the embryo and to optimize the conditions under which it is grown to ensure the best start in life. Metabolic studies allow us to gain vital information on the requirements of a competent embryo and identify when things go wrong, but the reader is cautioned towards careful interpretation of measures of metabolism especially between laboratories and to consider the environment as a whole under which they have been taken.

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Appendix 2: Published paper demonstrating ‘proof of concept’ of IVP system protocols used over the course of the studies contained in this thesis

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Effect of oocyte source and transport time on rates of equine oocyte maturation and cleavage after fertilization by ICSI, with a note on the validation of equine embryo morphological classification

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Abstract

Production of equine embryos using intracytoplasmic sperm injection (ICSI) is rapidly gaining interest in the horse industry. Due to increasing client demand, equine practitioners with limited experience in embryology are attempting to set up clinical ICSI programs, with little success. We report here studies performed with the purpose of establishing an equine in vitro maturation (IVM)/ICSI program. We addressed three objectives: to determine (1) the effect of oocyte source (transvaginal follicle aspiration [TVA] vs. abattoir) on maturation, cleavage and blastocyst rates; (2) the impact of time of oocyte recovery (soon after death vs. delayed [median time 7.5 h]) on these parameters in abattoir-derived ovaries; and (3) the correlation of post-ICSI embryo morphology with histologically-confirmed nuclear status. Maturation rates were greater for TVA-derived than for abattoir-derived oocytes (67% vs. 32%, respectively; $P < 0.01$). Duration of ovary transport did not affect oocyte maturation (34-48%) or cleavage rates (70-73%). Chromatin staining revealed that light-microscopic evaluation was accurate in determining oocyte maturation to metaphase II (14/15, 93%), but was not accurate in classification of either normal embryo cleavage (8/31 cleaved embryos possessed normal nuclei) or blastocyst formation (7/15 embryos classified as blastocysts were verified on staining). Early embryo and blastocyst viability was confirmed by transfer of some embryos into recipient mares. These findings indicate that oocyte source (TVA vs. abattoir) can affect results in an equine IVM/ICSI system, and suggest that new laboratories should use a systematic approach of comparison of nuclear chromatin staining with morphological classification to validate embryo development after ICSI.

Keywords: Horse, ICSI, blastocyst, oocyte, embryo

Introduction

The ability to produce foals using ICSI has the potential to improve our understanding of early embryo development and has clinical applications in the salvage of valuable equine genetics.¹ In mares, this is either following post-mortem oocyte recovery or by collecting oocytes from living sub-fertile mares that cannot provide an embryo for transfer^{2,3} and in stallions, by offering a mechanism to produce embryos and foals when only limited reserves of semen are available.

The *in vitro* production of viable blastocysts and subsequent pregnancies, encompasses many complex and interdependent procedures, as well as requiring knowledge of cell culture and embryological techniques, such that the development of a successful equine ICSI program has been described as “practically unattainable in most situations”.⁴ Interest in the use of equine ICSI in both commercial and research applications has rapidly increased worldwide, and client demand has resulted in an increasing number of equine practitioners and equine laboratories with limited experience in *in vitro* embryo production embarking on the establishment of commercial equine ICSI laboratories. Given the challenges, successes have been limited. Groups that report blastocyst development after equine ICSI typically achieve a $< 10\%$ blastocyst rate per injected *in vitro*-matured (IVM) oocyte.⁵⁻⁸ Although it is generally understood that equine ICSI is feasible, in reality, only a few laboratories have reported high blastocyst rates ($>20\%$) using IVM oocytes⁹⁻¹² and only two of these have reported $>20\%$ blastocyst rates for this procedure more than once ($\sim 25-43\%$).^{3,11-15}

The rate of oocyte maturation is another major determinant of commercial success. If blastocyst rates are low ($<10\%$), production of one blastocyst would take over ten mature (metaphase II [MII]) oocytes. For many laboratories, oocyte recovery and *in vitro* maturation rates currently conspire against this. Rates of *in vitro* maturation are variable (44 to 61%), particularly when collecting oocytes post-mortem, as in genetic salvage cases.^{11,16}

Clinically, in the case of genetic salvage, a mare often dies or is euthanized at a location distant from the ICSI facility. Under these circumstances ovaries/oocytes must be optimally retrieved and transported to the ICSI facility. Despite anecdotal reports, currently, only a single report has

detailed the clinical production of foals by ICSI under these conditions. In that report, pregnancies were obtained following ovary removal at the location of death and transport of both whole ovaries or recovered oocytes.²

Another aspect affecting development of an ICSI program is the ability of the practitioner to appropriately identify viable embryos. While some laboratories have validated their classification of embryos as blastocysts by chromatin staining for nuclear status or by achieving high pregnancy rates (>50%) after transfer, some laboratories have assessed blastocyst development based only on morphological appearance.^{3,6,12,17,18} The morphology of *in vitro* produced (IVP) equine blastocysts differs from both the morphology of IVP blastocysts in other species and from that of equine *in vivo*-derived blastocysts, thus identification of viable embryos may be problematic for the inexperienced eye. However, to the best of our knowledge, other than early reports from the laboratory in Texas which reported both morphological cleavage and cleavage with normal nuclei (41% to 88% of morphologically cleaved embryos had normal cleavage on nuclear evaluation),¹⁹⁻²¹ essentially no information is available on the relationship of light-microscopic morphological classification to actual embryo status as shown by evaluation after nuclear staining.

In this study, we performed three evaluations to establish basic parameters for the development of an equine ICSI program. We determined (1) the effect of oocyte source (TVA from live mares vs. abattoir-derived) on maturation, cleavage and blastocyst rates after ICSI; (2) the relative impact of oocyte recovery soon after death as opposed to a delay following the transport of entire ovaries and (3) the correlation of post-ICSI embryo morphology on light microscopy with histologically-confirmed nuclear status.

Materials and methods

All procedures were conducted in agreement with the principals of the University of Liverpool Veterinary Ethics Committee and with the approval of the Royal College of Veterinary Surgeons.

Oocyte recovery from abattoir-derived ovaries

Ovaries were obtained from mares, of unknown age and breed, slaughtered at a UK abattoir for purposes unrelated to the study. Slaughter was conducted in accordance with EU legislations EC 852/2004, 853/2004 and 854/2004. Ovaries were obtained within 15 min post-mortem, placed in a polythene bag within a polystyrene foam container and maintained at ambient temperature (18-20°C) until all tissues had been processed. Ovaries were either transported to the ICSI laboratory (transport time 4 hr) or to a local facility as dictated by the study protocols detailed below. On arrival, all visible follicles were aspirated using a 14 G needle and vacuum pump (Rocket Medical PLC., Watford, Herts, UK) set at approximately 200 mmHg. All plastics (MILA international, Erlanger, Kentucky) were sterilized in ethylene oxide with a minimum gas-off time of 4 wk. A syringe and needle were used to flush each follicle one to two times with flush medium (M199 with Hanks salts, 0.4% Fetal Bovine Serum (FBS) (Life Technologies Ltd, Paisley, UK), 25 µg/ml gentamicin, 8 IU/ml heparin (Sigma-Aldrich Ltd, Gillingham, Dorset, UK). Ovaries were then sliced at 5-10 mm thickness and additional visible follicles were aspirated and scraped using the 14 G needle with vacuum. Aspirated fluid was collected into sterile 500-ml bottles and passed through an embryo filter (Emcare ICPbio Reproduction, Auckland, NZ). The filter was rinsed with flush medium and cumulus-oocyte complexes (COCs) were located in the recovered tissue under a dissection microscope at 60-120X. Identified COCs were moved to manipulation medium (M199 with Hanks salts, 10% FBS, 25 µg/ml gentamicin). All manipulations were conducted at ambient temperature (approximately 18-20°C).

Oocyte collection from live mares

Oocytes were recovered by TVA from six sport-horse type mares aged 7-14 years. The TVA procedures were conducted as previously described.¹⁰ All visible follicles > 0.5 cm were aspirated; each follicle was flushed up to six times with flush medium. The aspirated fluid was processed within 1-2 hr of aspiration, as described above for fluid aspirated from follicles in abattoir-derived ovaries.

Oocyte holding

Recovered COCs were divided into groups of eight to 14 and placed in individual 1-ml glass vials (Thermoscientific Inc., Waltham, MA) in EH Medium (40% M199 with Hanks salts, 40% M199 with Earle's salts, 20% FBS and 25 µg/ml gentamicin).²² Vials were sealed and held overnight (12-18 hr) at room temperature (20°C) protected from light.

Oocyte maturation

Maturation dishes (pre-prepared with medium and oil) were pre-equilibrated for 12 hr before use. Cumulus-oocyte complexes were removed from the EH holding vial, washed in maturation medium (M199 with Earle's salts, 10% FBS, 25 µg/ml gentamicin with 5 mU/ml FSH [Sioux Biochemical Inc., Sioux Center, IA]) and incubated in groups of 9-12 at a ratio of 10 µl medium per COC in a 4-well dish (Thermoscientific), under mineral oil (Vitrolife Ltd., Warwick, UK) for 30 hr at 38.3°C in 5% CO₂ in air. Oocytes were then denuded of cumulus cells by repeated pipetting in manipulation medium containing 80 IU/ml hyaluronidase (Vitrolife). Oocytes were evaluated by light microscopy using a dissection microscope at 500X. Those with a visible polar body were classified as metaphase II (MII), oocytes with an intact oolemma without a polar body were classified as intact and those with an irregular oolemma or shrunken cytoplasm were classed as degenerating (DEG). Confirmation of correlation of oocyte morphological classification with chromatin status was performed in experiment 3.

ICSI and Embryo Culture

Frozen-thawed sperm from one fertile stallion was used for ICSI. For each ICSI session, a single 0.5-ml straw of semen was thawed at 37°C for 30 sec. To perform swim-up, 200 µl of thawed semen was placed under 3 ml of frozen-thawed Sp-Talp²³ and incubated at 37°C for 30 min. Sperm suspension from the top of the Sp-Talp layer was used for ICSI.

Conventional ICSI was performed using an Integra micromanipulator (Research Instruments, Falmouth, UK) by an embryologist (KS) with extensive experience in human clinical ICSI. A standard sharpened ICSI needle (5 µm inner diameter; Research Instruments) was used. Denuded MII oocytes were placed in individual 10-µl droplets of manipulation medium and 1-2 µl of sperm suspension was placed in a separate 5-µl droplet of a commercial 9% PVP solution (ICSITM; Vitrolife). Motile spermatozoa were immobilized by crushing of the flagellum with the ICSI needle until kinking was observed and aspirated into the ICSI needle flagellum first. The oocyte was positioned with the polar body at 6 o'clock and the ICSI needle was advanced through the zona pellucida and into the oocyte cytoplasm, essentially to the opposite side of the oocyte. Puncture of the oolemma was confirmed by visualization of cytoplasmic contents in the ICSI needle after suction and the spermatozoon was then deposited in the cytoplasm with a minimum of medium. Micromanipulator platform temperature was controlled in relation to ambient temperature in an attempt to perform all ICSI manipulations at 37°C.

Injected oocytes were washed twice in pre-equilibrated culture medium (DMEM-F12 [Sigma Aldrich] with 10% FBS and 25 µg/ml gentamicin)¹² to remove manipulation medium and were placed in 20-µl droplets of culture medium under oil (four to ten injected oocytes/droplet, i.e. 2-5µl per injected oocyte) in a 4-well dish, (Thermoscientific) at 38.3°C in 5% CO₂, 5% O₂ and 90% N₂. Except when noted below, embryos were assessed for cleavage on Day 3 (Day 0 = Day of ICSI), at which point uncleaved oocytes were moved to a different droplet within the same dish. Medium in the original droplet was refreshed by adding 20 µl of fresh culture medium and then removing 20 µl. From Day 7 to Day 11, embryos were assessed for blastocyst development daily by visualization under an inverted microscope at 200X. Embryos were classified as blastocysts morphologically based on an increase in diameter from the previous day and a multicellular appearance with presence of an apparent uniform outer cell layer.

Transcervical embryo transfer

Embryos were loaded into 0.25 ml straws using pre-equilibrated culture medium, warmed to 38.3°C. A stainless steel gun with a disposable sheath (both IMV Technologies, France) was used for the transfer. Altrenogest (Regumate, MSD Animal health, Buckinghamshire, UK; 0.044 mg/kg bwt PO q 24 hr) was administered to each recipient mare beginning on the day of transfer. Pregnancy

diagnosis was first performed via transrectal ultrasonography on Day 14 (Day 0= Day of ICSI). If an embryonic vesicle was not present, examinations were repeated every 2-3 days until Day 20 at which point the mare was declared non-pregnant and altrenogest treatment was discontinued. If an embryonic vesicle was visualized on one of these examinations, examination was repeated on days 21, 28, 45, 60 and 90. Altrenogest treatment was continued until Day 120.

Oviductal embryo transfer

Transfer of embryos to the oviduct was performed by flank laparotomy under standing sedation as described previously,²⁴ with the following major modifications: 1) a 15-cm oblique incision was made in the paralumbar fossa contralateral to the site of ovulation, beginning just ventral to the cranioventral aspect of the tuber coxae and extending cranially and ventrally perpendicular to the last rib; 2) injected oocytes/embryos were loaded into a modified 6 F dog urinary catheter attached to a 1 ml all-plastic syringe with < 0.5 ml of culture medium for transfer.

Evaluation of oocytes and embryos for chromatin status

Oocytes or presumptive embryos were briefly fixed in buffered formal saline and mounted on a slide with 7 µl mounting medium (9:1 glycerol:PBS) containing 5 µg/ml Hoechst 33258 and examined by fluorescent microscopy (range 350-461nm) at 400X. Oocytes were classified as MII, MI, GV (having an intact nucleus) or degenerating (no chromatin or abnormal chromatin). Zygotes were evaluated for presence of two pronuclei and two polar bodies. Cleaved embryos were evaluated to determine the number and status of nuclei. Only nuclei that appeared to be normal were included in the number of nuclei recorded; nuclei with signs of degeneration (vacuolization, condensation or fragmentation) were disregarded. Embryos were confirmed to be blastocysts if they contained more than 64 normal nuclei and showed arrangement of an outer rim of nuclei in a presumptive trophoblast layer.

Preliminary study. To establish oocyte collection and maturation procedures, ovaries were obtained from the abattoir and transported to the main laboratory for processing. After EH holding, oocytes were placed in culture in maturation medium as described above, then evaluated morphologically for presence of a polar body after 30 hr culture.

Experiment one: the effect of oocyte source (abattoir vs. in vivo) on maturation, cleavage and blastocyst rates after ICSI. Oocyte maturation rates recorded in the preliminary study were low. This study was conducted to determine whether the oocyte source affected maturation rates. Oocytes were obtained from abattoir-derived ovaries as described above, or from live mares via TVA. After IVM, the proportion of oocytes having polar bodies was recorded for each treatment, and these oocytes were then subjected to ICSI. Injected oocytes were cultured, and evaluated for blastocyst development from Day 7 to Day 11. Embryos classified morphologically as having developed to blastocyst were either transferred transcervically to the uterus of a recipient mare, or were stained for evaluation of chromatin status.

Experiment two: effect of ovary transport time on maturation, cleavage and blastocyst rate after ICSI. The maturation rate was higher with *in vivo*-derived oocytes in Experiment 1. It was hypothesized that the low maturation rates found in abattoir-derived oocytes in Experiments 1 and 2 might be related to the delay during transport of the ovaries to the laboratory. To test this hypothesis, abattoir-derived ovaries were divided into two treatments. Group 1: local facility (LOC) ovaries collected from mares slaughtered from 7 am to 1 pm were processed at a facility near the abattoir. Recovered oocytes were placed into EH medium as described for overnight holding above, then transported to the laboratory. Group 2: LAB, ovaries from mares slaughtered from 1-5 pm were collected and transported to the main laboratory for processing. Recovered oocytes were placed into EH medium for overnight holding.

After IVM and ICSI, injected oocytes were examined from Day 7 to 11 of culture for development to blastocyst, at which time all presumptive embryos were stained for evaluation of nuclear status. In one replicate, for the purpose of evaluating embryo viability, at 66 hr after ICSI, the embryos showing the greatest degree of development (uniform morphological cleavage and > six

cells) as assessed under light microscopy were transferred to the oviduct of a recipient mare on Day 1 after ovulation.

Experiment three: correlation of embryo morphology with nuclear status. Staining of embryos at Days 7 to 11 in Experiments 2 and 3 suggested that morphological classification was not accurately reflecting the nuclear status of embryos. To improve our understanding of the association between equine embryo morphology and nuclear status, a systematic, sequential embryo staining study was performed.

Oocytes recovered from abattoir-derived ovaries and processed at the main laboratory were matured *in vitro*, then subjected to ICSI and embryo culture as described above, with the modifications that 0.27mM pyruvate was added to the oocyte injection medium and the sperm preparation method was changed to direct swim up.¹⁰

Staining and assessment of chromatin status was performed at the following times: A) immediately after IVM in oocytes classified as MII on the basis of presence of a polar body; B) at 20 hours post-ICSI to evaluate pronuclear status; C) at Day 2, 3, 4, or 7 after ICSI to determine nucleus number and correlation of morphological cleavage classification with nucleus number; and D) after seven to 11 days of culture. In group D, embryos classified morphologically as blastocysts were stained and evaluated on the day they were identified, then all remaining presumptive embryos were stained and evaluated on Day 11 of culture. Two Day-9 presumptive blastocysts from this group were not stained but were transferred to the uterus of a recipient mare five days after ovulation to evaluate viability.

Statistical analyses

Differences in rates of oocyte maturation, cleavage, and blastocyst development were compared among groups by Chi-square analysis, with Fisher's Exact Test used between treatments when a value of less than ten was anticipated for any parameter.

Results

Preliminary study

Five replicates were performed, and 49 oocytes were recovered and subjected to IVM. The rate of maturation to MII was 39%.

Experiment one: effect of oocyte source (abattoir vs. *in vivo*) on maturation, cleavage and blastocyst rates after ICSI

Fifteen TVA procedures were performed, and 99 oocytes were recovered (Table 1). Three replicates were performed with abattoir-derived ovaries. Overall maturation rates were greater for oocytes obtained by TVA than for oocytes from abattoir-derived ovaries (67 % vs. 32%, respectively; $P < 0.01$). There was no significant difference between groups in morphological cleavage rate after ICSI (50 -53 %).

On Day 8 after ICSI, on visual appraisal, based on increased diameter and the apparent presence of a uniform outer cell layer, four embryos (two TVA and two abattoir), were considered to have developed to the blastocyst stage. Three of these were transferred transcervically (one single [abattoir]; one double [TVA]) to the uteri of two recipient mares six days after ovulation. No pregnancies resulted. The fourth presumed blastocyst was stained with Hoechst 33258; on evaluation, this proved to be a degenerated embryo.

Experiment two: effect of ovary transport time on maturation, cleavage and blastocyst rates after ICSI

Three replicates were performed (264 oocytes). The time from mare death to placement of oocytes in EH media for the LOC treatment ranged from 0.5- 4 hr (median 2.25 hr); in the LAB treatment this ranged from 5-10 hr (median 7.5 hr). Three metaphase II oocytes were lost during manipulation and not injected. There was no significant difference in oocyte maturation or cleavage rates between LOC and LAB treatments (Table 2).

Transfer of the four most advanced embryos at 66 hr culture in one replicate resulted in two embryonic vesicles (4 mm and 5 mm in diameter) detected by transrectal ultrasonography of the recipient mare on Day 17 after ICSI. These vesicles were lost by Day 24. None of the 61 cleaved

embryos cultured to Day 11 were classified as blastocysts morphologically. However, on evaluation after staining, one blastocyst was identified in this group based on the presence of > 64 cells and an organized outer layer of nuclei.

Experiment three: correlation of embryo morphology and nuclear status

Staining and evaluation of chromatin status was performed at sequential steps to validate morphological classification of oocyte and embryo status. In one replicate, 18 oocytes presumed to be in MII based on presence of a polar body were stained to confirm meiotic status; three were lost during manipulation and 14/15 (93%) were confirmed in MII (Fig. 1A). A total of 109 oocytes were subjected to ICSI. Over four replicates, the rate of pronuclear (PN) formation at 20 hr after ICSI was 43% (10/23 oocytes; Fig. 1B); a further eight injected oocytes were lost during processing. Over six replicates, 46 presumptive embryos were stained on Day 2, 3, 4, or 7 after ICSI. Of these, 31 (64%) demonstrated apparent morphological cleavage, but only eight of these (17%) had two or more normal nuclei on staining. In 3/46 embryos (6.5%), the number of nuclei matched the number of visible blastomeres and were appropriate for age (Fig. 2A); five embryos possessed two to six normal nuclei, but nucleus number was lower than expected for day.¹⁶ The other 23 stained embryos that appeared cleaved morphologically possessed only degenerated nuclei or were completely anuclear (Fig. 2B).

In five replicates, presumptive embryos were cultured to determine blastocyst development (Table 3). Seven metaphase II oocytes were used in other studies and not injected. After culture for 7 to 11 days, 17 presumptive embryos were classified as having developed to the blastocyst stage based on morphological criteria. Of these, 15 were stained for nuclear evaluation and two were transferred to the uterus of a recipient mare (results below). Evaluation of the 15 presumptive blastocysts after staining revealed seven confirmed blastocysts (e.g. Fig. 3A1-3, Fig. 4) and eight degenerating embryos (e.g. Fig. 3C1-2). In these replicates, at Day 11 all remaining structures (not classified as blastocysts) were stained, and one of these was also revealed to be a blastocyst (Fig. 3D1-2). Notably, as uncleaved oocytes were placed in a separate droplet at Day 4 but were kept in culture, we could evaluate changes in these oocytes over time. Several known uncleaved oocytes increased in diameter on Day 9 (e.g. Fig. 3B1), which, on simple morphological evaluation, could have led to mistaken classification of these structures as blastocysts (Fig. 3B2-3).

Transcervical transfer of two Day-9 presumptive blastocysts in the final replicate, to the uterus of a recipient mare five days after ovulation resulted in two embryonic vesicles (3 mm and 10 mm in diameter) detected by transrectal ultrasonography on Day 14 after ICSI. The smaller vesicle was manually reduced at Day 16 and the remaining vesicle developed normally. A healthy colt foal was born at 345 days gestation (Fig. 5). Overall, in these five replicates, including the two transferred embryos, the rate of “blastocyst” development based on morphological assessment was 17/138 (12.3%); however, the confirmed blastocyst development per injected oocyte was 10/138 (7.2%).

Discussion

This study highlights factors that may affect the development of an effective equine ICSI program. We found an increased rate of meiotic maturation in oocytes derived from live mares by TVA compared to oocytes recovered post-mortem. The maturation rate of post-mortem derived oocytes was not affected by ovary transport time (up to 10 hr). Notably, when assessing embryo development in the first two experiments, we found discrepancies between our morphological classification of the embryos on light microscopy and the findings on staining for nuclear evaluation. This led us to evaluate the accuracy of our morphological classifications via step-wise oocyte and embryo staining in Exp. 3.

The rate of nuclear maturation in our preliminary study with abattoir-derived oocytes was low (39%). To determine if this was related to oocyte source, or to our untested IVM system, we compared maturation rates of TVA vs. abattoir-derived oocytes. This showed that with TVA oocytes the IVM system produced acceptable rates of maturation to MII (67%) whereas maturation remained low for oocytes from abattoir-derived oocytes, serving to validate the IVM system. We then evaluated whether the long transport time was associated with the low maturation rates in abattoir-derived oocytes; however, maturation remained low for oocytes collected after a median time of 2.25 hr or 7.5 hr. It is possible that changes in oocyte meiotic competence had already occurred within our

shortest handling duration (2.25 hr in the LOC group), thus lowering the maturation rates. A study by Guignot et al. also found no difference in maturation rates across transport times (1.5- 4 hr vs. 6-8 hr);²⁶ while Hinrichs et al. did find a higher maturation rate in oocytes collected < 1 hr of slaughter than in oocytes collected 5-9 hr after slaughter. Due to abattoir location and available processing facilities, the LOC group represented the most rapid processing time possible in this study. Of course, transport time is only one factor affecting rates of meiotic maturation and developmental competence in abattoir-derived ovaries, others including temperature of transport, mare age and health, and exposure of the ovaries to toxic compounds at the abattoir. Several studies have investigated optimal transport temperatures for equine ovaries and it was suggested that for periods < 1 hr, ovaries should be kept at 30-37°C but for longer periods, room temperature (22-24°C; as in this study) was more appropriate and resulted in the highest developmental competence.²⁷⁻²⁹

The final blastocyst rate (number of confirmed blastocysts per injected oocyte) achieved was 7.2%, which is similar to that reported by some established ICSI laboratories^{6,8} but lower than optimal (20-40%).^{3,10,30} All embryos in this study were produced by conventional ICSI. Historically, the first equine ICSI pregnancy was produced using conventional injection and surgical transfer of early embryos.³¹ Some laboratories applied chemical oocyte activation due to low cleavage rates after ICSI,¹ although soon after, workers employing Piezo drill-assisted ICSI indicated that chemical activation was unnecessary.¹⁹ On the basis of successes with the Piezo drill,^{12,21,33} it has been hypothesized that this technique is an important aid to equine ICSI. In the only study in which Piezo-assisted and conventional (laser-assisted) ICSI were directly compared, no significant difference was recorded, however, blastocyst rates per injected oocyte were low for both methods (4.8% and 5.1%, respectively).⁶ The same authors have reported a live foal produced following conventional ICSI.³⁴ That study, and ours report blastocyst development at a much lower rate (7.4 % and 7.2% respectively) than do those that use Piezo-assisted ICSI (> 25%),^{10,11,14} but clearly there are a myriad of other potential differences among these laboratories. There is a need for a systematic, prospective study within the same laboratory to establish whether Piezo-assisted ICSI offers a significant advantage over conventional ICSI. This is important, as the inclusion of the Piezo drill represents a significant additional investment for new laboratories and introduces an additional requirement for technical expertise.

Difficulties were experienced in correctly interpreting the morphology of cleaved embryos. Initially, we used a human grading system, which was shown not to be appropriate for application to equine embryos. The systematic staining of cleaved embryos revealed that only three of 31 embryos, empirically classified as morphologically cleaved, actually possessed the number of nuclei appropriate for age, and we found that many embryos contained anuclear blastomeres.²⁵ The significance of this latter finding is unclear. A recent study in humans reported that presence of anuclear blastomeres had no significant effect on birth rate.³⁵

Blastocyst identification was also problematic. Even when interpretation was based on images and information from laboratories working in equine ICSI,^{10,36} we still encountered discrepancies between morphological blastocyst appearance and nuclear status (Figure 3). It was noted that several known uncleaved oocytes increased in diameter between Days 7 and 9 after ICSI, and these were shown to have no nuclear chromatin upon staining (Figure 3B1-3). This phenomenon has not to the best of our knowledge been presented previously. Certain time-dependent cell-cycle and development stages can occur independently of nuclear status,³⁷ therefore it could be hypothesized that there are cytoplasmic developmental clock mechanisms driving oocyte expansion in the absence of cleavage. Our findings reinforced the importance of removing uncleaved oocytes to limit uncertainty in later assessments of blastocyst development, and of staining embryos for DNA to definitively establish blastocyst development. In practical terms, chromatin integrity cannot be evaluated in presumed blastocysts designated for transfer. Here, the benefits gained from coupling morphological assessments with definitive evaluations of nuclear status were invaluable. Morphology can be used to reliably select true blastocysts, as shown by the high initial pregnancy rate of IVP blastocysts after transfer reported by experienced laboratories (82/101, 82%;³⁰; 9/13, 69%;³). In our case, morphological selection of two blastocysts in the final replicate of the study resulted in two embryonic vesicles.

To the best of our knowledge, this is the first report to document the disparity between morphological and histological assessment in equine IVP embryos. When setting up an ICSI

laboratory, it is tempting, as we did in Experiments 1 and 2, to transfer structures presumed to be embryos to recipient mares in an attempt to establish pregnancies and thus validate the program. However, in retrospect, this was counter-productive as evaluating morphology alone proved to be inaccurate and misleading. Our data and the related figures from Exp. 3 highlight the need for objective verification of embryo status. We hope that by sharing our experience of identifying discrepancies between morphological classification and actual embryo developmental stage, we have highlighted that morphology must be confirmed by a method such as nuclear staining when laboratories are initially evaluating their rate of blastocyst production.

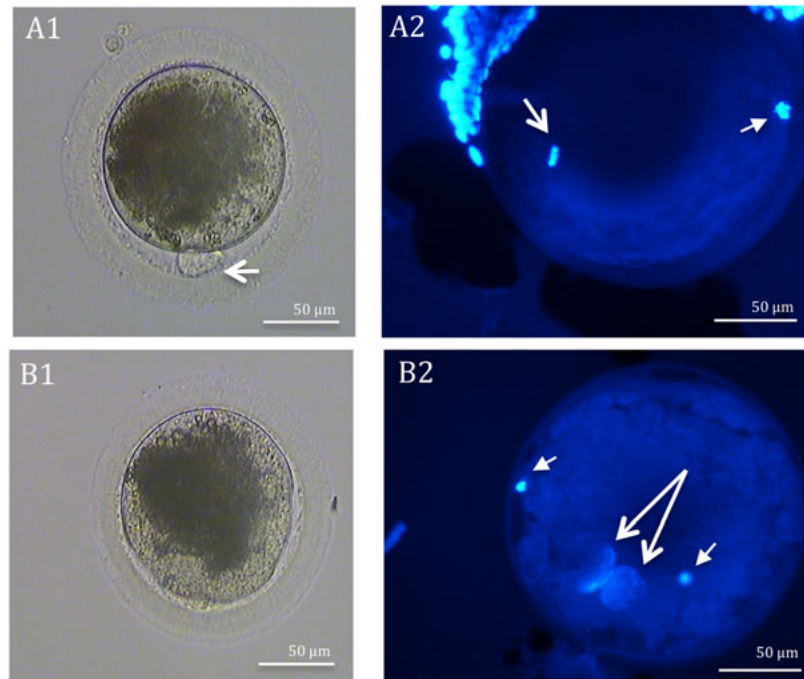


Figure 1: Photomicrographs of oocytes evaluated after IVM to validate MII status (A) and after ICSI to validate fertilization status (B). A1, B1: bright field microscopy 300X; A2, B2: after staining with Hoechst 33258, under fluorescence 400X. A1: MII oocyte with polar body (arrow); A2: metaphase plate (arrow) and polar body (closed arrow); B1: Injected oocyte 22 hours after ICSI B2: Fertilization confirmed by visualization of two pronuclei (staining incomplete; arrows) and two polar bodies (closed arrows).

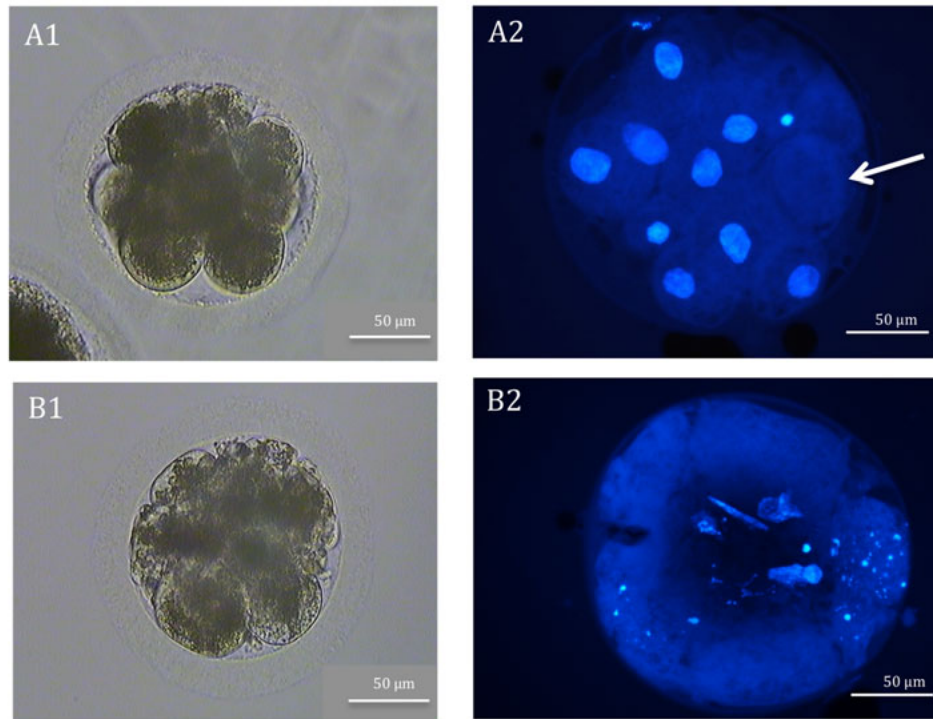


Figure 2: Photomicrographs of two embryos 96 hours after ICSI. A1, B1: bright field 300X; A2, B2 after staining with Hoechst 33258 400X. A1: apparent 10-cell embryo; A2: 9 normal nuclei and one anuclear blastomere (arrow); B1: apparent 8-cell embryo; B2: no normal nuclei.

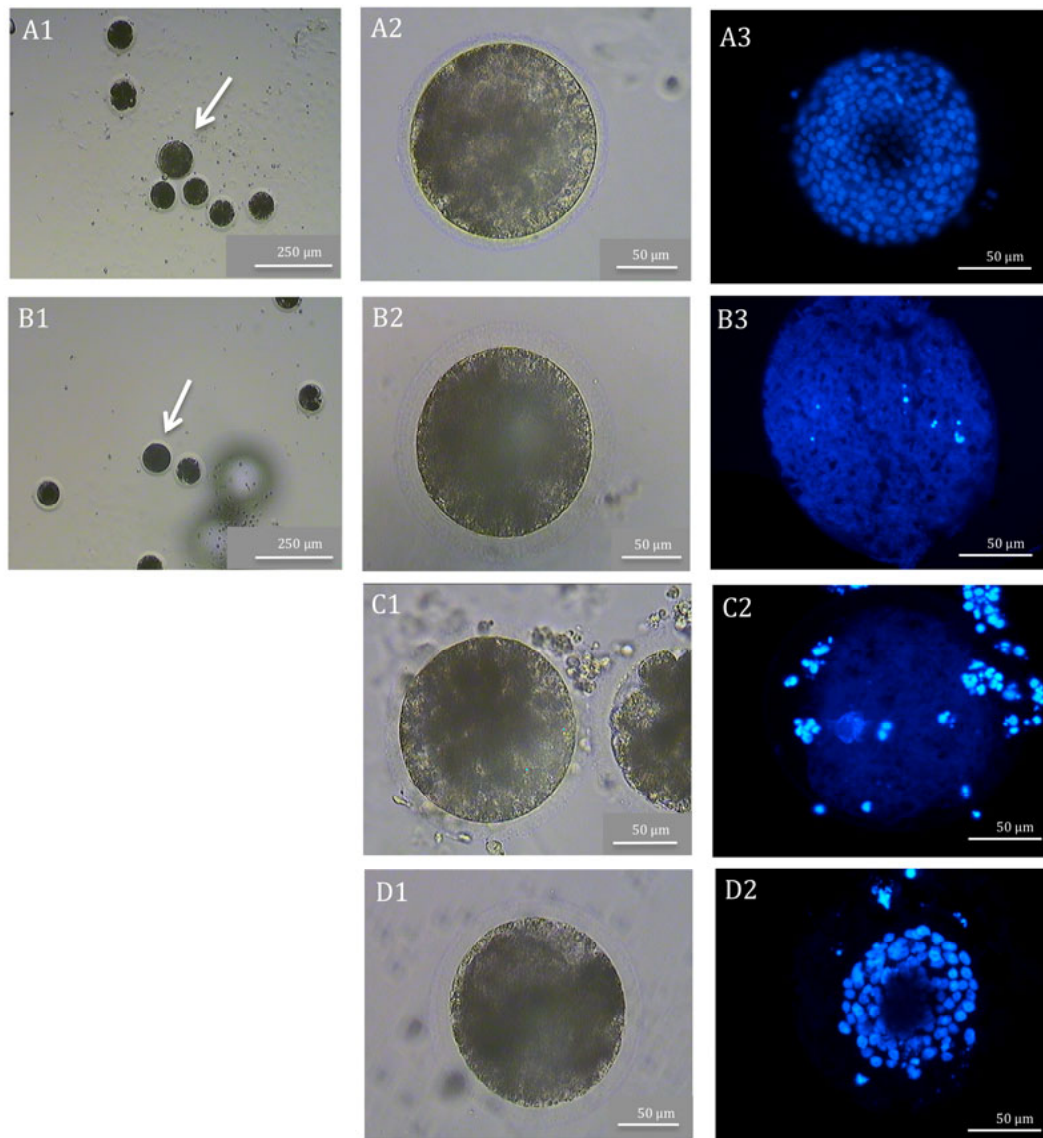


Figure 3: Photomicrographs of presumptive blastocysts at Day 9 after ICSI, showing discrepancies between morphological classification and nuclear status. A1 and B1: bright field images (60X) of presumptive embryos shown in panels A2-A3 and B2-B3, respectively, showing relative increase in diameter (arrows). B1-3; structure arising from uncleaved oocyte. Left: bright field (300X); right, after Hoechst 33258 staining (400X). A2: Presumed blastocyst shown in A1, with presumptive trophoblast layer (outer layer of organized cells) and thinning zona pellucida (ZP). A3: Status as blastocyst (presence of >64 cells with organized outer layer of nuclei) confirmed after staining. B2: structure pictured in B1, having similar morphology to blastocyst; B3: structure confirmed as a degenerating uncleaved oocyte, with no visible chromatin. C1: presumed blastocyst (from cleaved embryo) due to increase in size and thinning of ZP. C2: staining revealed structure to be degenerating. D1: presumed degenerating embryo (from cleaved embryo) due to irregular cytoplasmic outline and lack of size increase. D2: staining revealed structure to be blastocyst (penetration of the stain is incomplete).

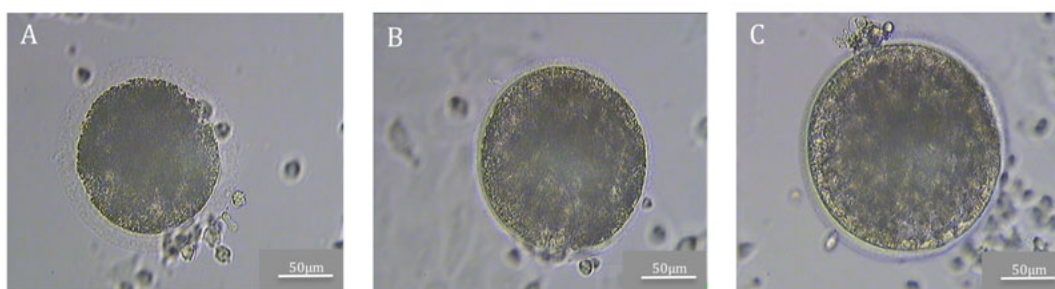


Figure 4: Photomicrographs (Bright field 300X) showing sequential development of a blastocyst on Days 7 through 9 (A-C) after ICSI (blastocyst status was subsequently confirmed by staining). An increase in diameter and in organization of the presumptive trophoblast layer can be seen.



Figure 5: Photograph of ICSI foal at 5 days old.

Editor's note: The photographs in this manuscript are available in color in the online edition of Clinical Theriogenology.

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Table 1: The effect of oocyte source (abattoir derived vs. TVA) on maturation, cleavage and blastocyst rates after ICSI. TVA= Transvaginal aspiration from live mares.
 * = Presumed status based on morphology. Day 0= day of ICSI. Mares were examined for pregnancy from Days 14-20.

	Replicates <i>n</i>	Ovaries <i>n</i>	Oocytes <i>n</i>	MII <i>n</i>	Injected <i>n</i>	Cleaved* <i>n</i>	Blastocysts* <i>n</i>	Embryos transferred (<i>n</i> , time post ICSI)	Pregnancies <i>n</i>
Abattoir derived	3	24	56	18 (32%) ^a	18	9 (50%)	2	1, Day 8	0
TVA	15	52 (26 mares)	99	66 (67%) ^b	66	35 (53%)	2	2, Day 8	0

Table 2: Effect of ovary transport time on maturation, cleavage and blastocyst rate after ICSI. * = Presumed status based on morphology. Blastocyst rates based on confirmation by DNA staining with Hoechst 33258 or pregnancy.

	Median time death- EH media (hr)	Ovaries <i>n</i>	Oocytes <i>n</i>	MII <i>n</i>	Injected <i>n</i>	Cleaved* <i>n</i>	Blastocysts <i>n</i>	Embryos transferred (<i>n</i> , time post ICSI)	Pregnancies <i>n</i>
Local facility (LOC)	2.25	114	166	57(34%)	57	40 (70%)	0	4 (66hr)	2
Laboratory (LAB)	7.5	86	98	41 (48%)	38	25 (66%)	1	0	0

Table 3: Results of Day 11 staining in experiment 3. Blastocyst rates based on confirmation by DNA staining with Hoechst 33258 or pregnancy.

Ovaries <i>n</i>	Oocytes <i>n</i>	MIII <i>n</i>	Injected <i>n</i>	Cleavage <i>n</i>	Morulae <i>n</i>	Presumed Blastocysts <i>n</i>	Confirmed Blastocysts <i>n</i>	Embryos transferred <i>n</i>	Day 14 Pregnancies <i>n</i>
214	326	145 (44%)	138	82 (59%)	3	17	10 (7.2%)	2	2

Appendix 3: Multivariable models to accompany results of Chapter 4

Experiment 4.1: Effect of oxygen concentration and pyruvate concentration during

IVM on GPL metabolism.

		Coef.	95% CI		P
Glucose consumption	Media	7.17	0.53	13.82	0.04
	Oxygen	-1.1	-7.22	5	0.72
	Media*oxygen	0.78	-7.56	9.13	0.85
	Trimming	5.19	1.16	9.23	0.01
	Constant	3.07	-4.92	11.06	0.45
Lactate production	Media	-1.7	-11.13	7.69	0.72
	Oxygen	-7.07	-15.71	1.56	0.12
	Media*oxygen	6.4	-5.46	18.26	0.29
	Constant	25.76	18.67	32.84	<0.001
Pyruvate production	Media	-1.47	-2.47	-0.48	0.01
	Oxygen	-0.52	-1.43	0.4	0.27
	Media*oxygen	1.15	-0.1	2.39	0.07
	Cumulus classification	0.49	-0.12	1.1	0.11
	Trimming	1.22	0.62	1.82	<0.001
	Constant	0.09	-1.11	1.29	0.88
Lactate: Glucose ratio	Media	-0.67	-1.19	-0.16	0.01
	Oxygen	-0.2	-0.69	0.3	0.43
	Media*oxygen	0.37	-0.28	1.02	0.26
	Trimming	-0.47	-0.78	-0.16	<0.001
	Constant	2.77	2.17	3.37	<0.001

Table A3.1: Multivariable linear regression model for effect of media (0 mM pyruvate vs. 0.15 mM Pyruvate) and oxygen concentration (5% vs. 21%) during IVM on glucose consumption, lactate and pyruvate production and lactate : glucose ratio. * Indicates interaction term. Constant represents a visually trimmed compact COC matured in control maturation media (0 mM pyruvate) at 21% Oxygen.

Experiment 4.3

Study 1: Temporal changes in GPL metabolism over the course of IVM in the presence of pyruvate

	Variable	Sub Category	Coef.	95% CI		P
Glucose Consumption	Time	0-10h	Ref.			
		10-20 h	4.31	0.95	7.66	0.01
		20-30 h	-5.17	-8.49	-1.85	0.002
	Batch	1	Ref.			
		2	-6.8	-12.48	-1.13	0.02
		3	5.09	0.69	9.5	0.02
		4	-6.43	-12.12	-2.75	0.002
		5	-5.81	-10.76	-0.85	0.02
		6	2.78	-1.9	7.47	0.24
	Constant		17.41	13.49	21.32	<0.001
Lactate production	Time	0-10h	Ref.			
		10-20 h	3.14	-0.61	6.88	0.1
		20-30 h	-8.28	-12.07	-4.48	<0.001
	Batch	1	Ref.			
		2	-2.67	-14.29	8.95	0.65
		3	-13.05	-22.08	-4.02	0.01
		4	-10.52	-20.16	-0.9	0.03
		5	2.89	-7.22	13.02	0.58
		6	-2.93	-12.51	6.63	0.55
	Constant		36.56	29.28	43.84	<0.001
Pyruvate production	Time	0-10h	Ref.			
		10-20 h	0.73	-0.44	0.59	0.78
		20-30 h	1.05	0.52	1.58	<0.001
	Batch	2	Ref.			
		3	-0.88	-1.81	0.05	0.06
		4	-0.32	-1.23	0.6	0.5
		5	0.21	-0.73	1.14	0.67
		6	0.35	-0.55	1.25	0.45
	Constant		1.52	0.81	2.23	<0.001

Table 2 continued

	Variable	Sub Category	Coef.	95% CI		P
Lactate: Glucose ratio	Time	0-10h	Ref			
		10-20 h	-0.21	-0.41	0.37	0.92
		20-30 h	0.29	-0.11	0.69	0.16
	Batch	1	Ref			
		2	1.17	0.38	1.96	0.004
		3	-0.98	-1.51	-0.46	<0.001
		4	0.84	0.26	1.42	0.004
		5	1.33	0.74	1.93	<0.001
		6	-0.3	-0.86	0.26	0.3
	Constant		1.93	1.45	2.41	<0.001

Table A3.2: Mixed random effect multivariate linear regression model for effect of time during IVM on glucose consumption, lactate and pyruvate production and lactate : glucose ratio. COC ID was added as a random effect to account for repeated measures over time. Constant represents COC in batch 2 during 0-10 hours of in vitro maturation.

Study 2: The impact of oocyte presence on GPL metabolism (intact COC vs. granulosa only).

	Variable	Sub-Category	Coef.	95% CI		P
Glucose consumption	Time	0-10h	Ref			
		10-20 h	-2.83	-7.93	2.27	0.28
		20-30 h	-3.04	-8.14	2.06	0.24
	Intact COC		2.34	-3.35	8.03	0.42
	Time*COC	0-10 h*COC	Ref			
		10-20h*COC	10.24	3.65	16.83	0.002
		20-30h*COC	-1.31	-7.86	5.25	0.7
	Constant		13.31	8.89	17.73	<0.001
Lactate production	Time	0-10h	Ref			
		10-20 h	3.91	-1.61	9.41	0.17
		20-30 h	3.82	-1.69	9.34	0.17
	Intact COC		17.61	9.64	25.58	<0.001
	Time*COC	0-10 h*COC	Ref			
		10-20h*COC	1.01	-6.04	8.07	0.78
		20-30h*COC	-9.18	-16.23	-2.12	0.01
	Constant		12.81	6.6	19.04	<0.001
Pyruvate production	Time	0-10h	Ref			
		10-20 h	-1.89	-2.85	-0.94	<0.001
		20-30 h	-1.78	-2.72	-0.84	<0.001
	Intact COC		-1.3	-2.36	-0.24	0.02
	Time*COC	0-10 h*COC	Ref			
		10-20h*COC	1.84	0.58	3.1	0.003
		20-30h*COC	2.75	1.49	4	<0.001
	Constant		2.66	1.87	3.45	<0.001
Lactate:glucose ratio	Time	0-10h	Ref			
		10-20 h	0.74	0.18	1.31	0.01
		20-30 h	0.34	-0.22	0.9	0.23
	Intact COC		0.72	0.31	1.42	0.04
	Time*COC	0-10 h*COC	Ref			
		10-20h*COC	-0.95	-1.68	-0.23	0.01
		20-30h*COC	0.06	-0.68	0.79	0.88
	Constant		1.22	0.7	1.75	<0.001

Table A3.3: *Mixed multivariate random effects linear regression model for impact of oocyte presence (intact COC vs. granulosa only) on glucose consumption, lactate and pyruvate production and lactate: glucose ratio. * Indicates interaction term. Constant represents granulosa only during 0-10 hours of in vitro maturation.*

Study 3: The impact of presence/absence of FSH on GPL metabolism.

	Variable	Sub-Category	Coef.	95% CI		P
Glucose consumption	Time	0-10h	Ref			
		10-20 h	-1.24	-5.94	3.45	0.6
		20-30 h	-8.16	-12.86	-3.46	0.001
	FSH present		-0.76	-6.44	4.91	0.79
	Time*FSH	0-10 h*FSH	Ref			
		10-20h*FSH	5.83	-0.37	12.02	0.07
		20-30h*FSH	2.68	-3.5	8.84	0.4
	Constant		16.98	12.65	21.3	<0.001
Lactate production	Time	0-10h	Ref			
		10-20 h	-4.6	-9.54	0.33	0.07
		20-30 h	-15.68	-20.68	10.69	<0.001
	FSH present		1.6	-5.11	8.31	0.64
	Time*FSH	0-10 h*FSH	Ref			
		10-20h*FSH	8.72	2.23	15.2	0.008
		20-30h*FSH	9.59	3	16.18	0.004
	Constant		26.16	21.07	31.26	<0.001
Pyruvate production	Time	0-10h	Ref			
		10-20 h	-0.81	-1.36	-0.27	0.004
		20-30 h	-0.39	-0.95	0.16	0.17
	FSH present		0.11	-0.65	0.86	0.79
	Time*FSH	0-10 h*FSH	Ref			
		10-20h*FSH	1.05	0.32	1.77	0.005
			1.38	0.64	2.11	<0.001
	Constant		0.99	0.43	1.55	0.001
Lactate:glucose ratio	Time	0-10h	Ref			
		10-20 h	0.79	0.07	1.52	0.03
		20-30 h	-0.004	-0.78	0.77	0.99
	FSH present		0.01	-0.76	0.79	0.97
	Time*FSH	0-10 h*FSH	Ref			
		10-20h*FSH	-0.59	-1.56	0.37	0.23
		20-30h*FSH	0.58	-0.45	1.61	0.27
	Constant		1.66	1.08	2.23	<0.001

Table A3.4: Mixed multivariate random effects linear regression model for impact of FSH presence (5mU/ml vs. 0mU/ml) on glucose consumption, lactate and pyruvate production and lactate:glucose ratio. * Indicates interaction term. Constant represents COC matured without FSH during 0-10 hours of in vitro maturation.